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Molecular Cloning and Sequencing of the 26S Region of Western Equine Encephalitis Virus Strain 71V-1658

By:

Fay L. Schmaltz, Les P. Nagata¹ and George Rayner
Medical Countermeasures Section

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¹ Corresponding Author

January 1997

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ABSTRACT

A 3100 bp cDNA clone (pcDW-12) of the 26S region of western equine encephalitis (WEE) virus strain 71V-1658 was identified by dot blot hybridization from a cDNA library. The missing 5' end was PCR-amplified and engineered into pcDW-12 to obtain a full length clone of the 26S region. The resulting construct (XH-7) was restriction mapped and completely sequenced on both strands. Only eleven of the sixty-three nucleotide differences resulted in amino acid changes when the sequence was compared to WEE strain BFS-1703 (Hahn *et al*, 1988). In addition, the high degree of conservation of the structural proteins was maintained when compared to the N-terminal sequence of the E1 and E2 proteins of the McMillan strain of WEE (Bell *et al*, 1983). The conserved nature of the structural proteins would indicate that one strain of WEE should be able to cross-protect against all WEE strains. A 2.2 kb fragment at the 5' end of the genome was also cloned and sequenced, and demonstrated high homology to the available sequences for eastern equine encephalitis (EEE) and Highlands J (HJ) viruses, adding further evidence that the entire 5' nonstructural region of WEE was derived from EEE. In summary, the genetic cloning and sequencing of the WEE 26S region marks the critical first step in the generation of a subunit vaccine to WEE.

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INTRODUCTION

Western equine encephalitis virus is an alphavirus which is serologically related to Sindbis (SIN), HJ, Fort Morgan, Buggy Creek and Aura viruses. It appears to be a recombinant virus between SIN and EEE viruses (Hahn *et al*, 1988). WEE is endemic in western North America and subtypes/varieties have been isolated from Argentina (AG80-646), Brazil (BeAr 102091) and the former Soviet Union (Y62-33). In nature, WEE is transmitted to man and horses by mosquitoes (*Culex tarsalis* is the principal vector) from its amplifying host or reservoir of wild birds. Infection of man from the endemic cycle has resulted in only a limited number of infections in recent years. However, in the past, major epidemics of WEE have been recorded. The most extensive epidemic occurred in the western United States and Canada in 1941, with 3,336 recognized human cases and 300,000 cases of encephalitis in horses and mules (Peters and Dalrymple, 1990).

The incubation period for WEE is between 5-10 days. As described by Peters and Dalrymple, 1990, the infection varies from mild fever with headache to aseptic meningitis and encephalitis. The disease usually begins with the sudden onset of fever, malaise, headache, nausea, vomiting, and occasionally, respiratory symptoms. Vertigo, photophobia, lethargy, abdominal pain and myalgia are common symptoms. After a few days the headache becomes more intense, and in severe cases, disorientation, stupor, tremors, paralysis or coma may develop. Fatal cases usually succumb during the first week. Infants and children are more susceptible, and the onset may be more abrupt, often associated with seizures and convulsions. Pathological reflexes vary, but commonly include weakness and hypoflexia, and children often display nuchal rigidity, disturbed sensorium, involuntary movements and paralysis. After 10 days, patients usually begin a gradual convalescence. Most adults will recover fully, although infants and children are at higher risk of developing permanent damage (Peters and Dalrymple, 1990; Horne *et al*, 1991).

The pathogenicity of WEE resembles that of EEE, although it is less neuroinvasive and neurovirulent in both humans and laboratory animals. The primary pathological findings are located in the central nervous system and include multiple foci of necrosis. The case infectivity

ratio has been estimated to be around 1:1150 in adults and drops to 1:58 in children and 1:1 in infants. The case fatality rate is between 3-8%, although the infectious dose is not known (Peters and Dalrymple, 1990). Of note, out of seven reported cases of laboratory-associated/acquired infections with WEE (associated with egg cultures, suckling mice and aerosols from lyophilized material), two deaths were recorded (Office of Biosafety, Laboratory Centre for Disease Control - LCDC, Canada). No direct human to human transmission of the virus has been recorded and the virus is not demonstrated in blood of man after the onset of disease (Peters and Dalrymple, 1990). Highlands J virus is antigenically related to WEE, and was previously referred to as the eastern variant of WEE. It does not cause clinical disease in humans or horses, although a case of encephalitis in a horse was attributed to HJ (Hayes and Wallis, 1977; Karabatsos *et al*, 1988).

Western equine encephalitis virus is an enveloped virus, containing a nucleocapsid (NC) which encases a single strand of positive-sense genomic RNA. The RNA is estimated to be near 12 kb in length, based on the size of the SIN virus genome (Strauss and Strauss, 1994). The nonstructural proteins are translated directly from the 5' two-thirds of the genomic RNA, while the structural proteins are translated from a 26S subgenomic mRNA encoded in the 3' end. The 26S region of WEE strain BFS 1703 has been cloned and sequenced (Hahn *et al*, 1988). The structural proteins (NC, E3, E2, 6K and E1) are processed by proteolytic cleavage from a single polyprotein. Apart from the NC, the other proteins are posttranslationally processed in the endoplasmic reticulum including glycosylation of the E1 and E2 proteins and the addition of lipid. Extrapolating from research with SIN and Venezuelan equine encephalitis (VEE) viruses, neutralizing domains were mapped primarily to the E2 protein, and to a lesser extent, to the E1 protein (reviewed in Roehrig, 1986).

An inactivated WEE vaccine is in Investigational New Drug (IND) status and requires a minimum of three injections as well as yearly boosters. Its major problem is the relatively poor and short-term immunogenicity to WEE that is produced. Currently, no antiviral to WEE has shown potential for treatment of infections, although a synthetic double stranded RNA, poly-ICLC, will be evaluated at DRES. In summary, a better vaccine needs to be developed for WEE. At the US Army Medical Research Institute Infectious Diseases (USAMRIID), research

is progressing towards the production of a live attenuated WEE vaccine via mutagenesis of an infectious clone of WEE virus. Live attenuated vaccines have proven to provide long-term immunity, and in many cases, protection is life-long. However, new types of problems can arise with these vaccines. The live attenuated VEE vaccine (TC83) has had trouble with reversion of the virus to a more virulent form (occurred in up to 20 % of the recipients of the vaccine - personal communication, J. Smith, USAMRIID). A second potential problem to be addressed is whether a live attenuated WEE virus will interfere with live attenuated VEE or EEE components of a trivalent vaccine. As an alternative, USAMRIID is looking at a baculovirus-produced subunit vaccine and microencapsulation for enhanced delivery of inactivated vaccine.

At DRES, research has focused on the development of subunit vaccines to WEE. Subunit vaccines involve inoculation of individuals with the protective component of the virus (envelope proteins). Subunit vaccines are generally safer than live vaccines, causing fewer complications during vaccination and no risk of reversion. They can be produced in a variety of expression systems (i.e. yeast, vaccinia, or as plasmid DNA), eliminating the need for high level biocontainment production facilities. Although multiple doses are usually required, recent advances in microencapsulation and DNA immunization have allowed the administration of a single dose which can induce a protective immune response. Liposomal-encapsulation, in conjunction with aerosol delivery, could prove effective in enhancing the immune response (Wong *et al*, 1992; Saravolac *et al*, 1996). The first step in the construction of a recombinant vaccine is to clone the relevant sequences from the viral genome. In this paper, we describe the steps in the cloning and DNA sequencing of the 26S region of WEE virus strain 71V-1658.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO), Aldrich (St. Louis, MO) and Fisher Scientific (Edmonton, AB). Restriction and DNA modification

enzymes were purchased from Gibco/BRL (Burlington, ON), Pharmacia (Baie d'Urfé, PQ) or New England Biolabs (Beverly, MA). Other suppliers include: GeneAmpTM polymerase chain reaction (PCR) reagents purchased from Perkin Elmer (Foster City, CA); MultiprimeTM random primer labelling kits and radioisotopes from Amersham (Oakville, ON); avian myeloblastosis virus (AMV) reverse transcriptase from Life Sciences (Petersburg, FL); tissue culture media, sera, E-linkTM kit and PhotogeneTM reagent from Gibco/BRL (Burlington, ON); and bacteriological media (Difco, Detroit, MI; Gibco/BRL, Burlington, ON). DNA primers were synthesized and gel purified at the Regional DNA Synthesis Laboratory (Calgary, AB) based on the published WEE 26S sequence (Hahn et al, 1988).

VIRUS CULTURE AND PURIFICATION

Tissue culture was maintained in accordance to established practices (Bird and Forrester, 1981). BHK-21 and Vero cells were obtained from American Type Culture Collections (ATCC - Rockville, MD) and USAMRIID. All manipulations of WEE were performed following Biocontainment Laboratory level 3 (BL-3) requirements at USAMRIID. A 10% suckling mouse brain (SMB) suspension of WEE strain 71V-1658 was kindly provided by Dr. Nick Karabatsos, Centers for Disease Control (CDC), Fort Collins, CO. A seed stock of 71V-1658 was made by inoculation of 4 roller bottles of Vero 19 cells (USAMRIID) with 70 µl total volume of the SMB suspension at a multiplicity of infection (MOI) of less than 0.1. Each roller bottle of cells (90-95% confluent) was incubated with the virus in 10 ml of Earles minimal essential media (EMEM) supplemented with 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin containing 5% fetal calf serum (FCS) - 5% EMEM. Incubation was continued for 1 hr at 37°C and an additional 40 ml of 5% EMEM was added to each roller bottle. The cytopathic effect (CPE) was allowed to proceed to 80-85% (approximately 3 days). The lysate was harvested, debris removed by centrifugation at 10,000 x g, and the supernatant aliquotted and stored at -70°C. Virus stocks were prepared by infecting 20 roller bottles with the WEE seed stock at a MOI of 10. After cell lysis (80-85% CPE), the virus was precipitated from cleared supernatant by the addition of polyethylene glycol (PEG) MW 6000 to 7%(w/v) and NaCl to 2.3%(w/v). The virus suspension was gently stirred for 15-20 hr at 4°C, followed by centrifugation at 10,000 x g for 30 min at room

temperature (RT). The pellets were resuspended in a total volume of 10 ml of phosphate-buffered saline (PBS - 0.01 M sodium phosphate buffer, 27 mM KCl, 0.137 M NaCl, pH7.4), and 5 ml of virus suspension was carefully layered onto 30 ml of a 20-60%(w/w) continuous sucrose gradient in a SW 28 tube (Beckman, Mississauga, ON). The tube was centrifuged at 100,000 x g (27,500 RPM in SW 28 rotor) for 3.5 hr at 4°C. The virus was harvested by removal of the virus band with a pasteur pipette. The virus suspension was then diluted at least 1:3 with Hanks Buffered Saline Solution (HBSS - Bird and Forrester, 1981), and centrifuged at 100,000 x g for 1 hr at 4°C. The virus pellet was resuspended in HBSS or PBS (5-10 ml), aliquotted and stored at -70°C.

ISOLATION OF RNA

Viral RNA used in library constructions to HJ strain B320 and WEE strain 71V-1658 were a kind gift of Dr Dennis Trent, CDC (Fort Collins, CO). Subsequent batches of RNA were prepared from WEE virus strain 71V-1658 cultured on Vero or BHK-21 cells. The virus was lysed by incubation in 0.5 % sodium dodecyl sulfate (SDS) and RNA extracted using the cesium chloride (CsCl)/guanidium isothiocyanate method as described (Sambrook *et al*, 1989), with the RNA being pelleted through a 5.7 M CsCl cushion. The RNA was aliquotted, precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol (International Biotechnologies Inc. - IBI, New Haven, CT), then stored at -70°C. Prior to use, the RNA was pelleted 15 min at 11,600 xg in a microfuge at 4°C, washed with 80%(v/v) ethanol and dried in a Savant Speed-Vac AES 1000 (Savant Instruments, Hicksville, NY). The pellet was dissolved in nuclease-free water (Promega, Madison, WI; Gibco/BRL, Burlington, ON), and aliquots were stored at -70°C. Integrity of the RNA was checked on formaldehyde agarose gels as described by Sambrook *et al*, 1989. Aerosol-free, RNase-free pipette tips (Gordon Technologies, Mississauga, ON) siliconized RNase-free microfuge tubes, use of diethylpyrocarbonate (DEPC)-treated dH₂O for buffers and aseptic techniques were used for all manipulations and reactions. Gel boats were cleaned with 70%(v/v) ethanol and DEPC-treated water immediately before use.

cDNA LIBRARY CONSTRUCTION

Libraries to HJ strain B320 or WEE strain 71V-1658 were made by Invitrogen (San Diego, CA) using the following protocol: an oligo-dT primer was used for reverse transcription of 5 µg of viral RNA to cDNA. After synthesis of the second DNA strand, linkers were added and the dsDNA was size selected to greater than 2,000 bp. The sized, electroeluted DNA was ligated into the *Bst*XI site of prepared pcDNAII vector, and electroporated into electrocompetent DH1αF' *Escherichia coli* cells. The cells were amplified by growing on LB agar containing 50-100 µg/ml ampicillin.

MANIPULATION OF DNA

Well publicized methods were used for handling and manipulation of DNA (Sambrook *et al*, 1989; Ausubel *et al*, 1995). These include the use of aseptic technique for the handling bacteria and tissue culture. Where possible, high grade reagents, nuclease-free water (Promega, Madison, WI), siliconized microfuge tubes, sterile solutions and disposables were used. Phenol/chloroform extractions used an equal volume of a mixture of water-saturated distilled phenol (Gibco/BRL, Burlington, ON), chloroform and isoamyl alcohol in a ratio of 25:24:1 ratio. This was followed by ethanol precipitation using 1/10 to 1/20 volume sodium acetate (3 M) and 2-2.5 volumes molecular biology grade ethanol (IBI, New Haven, CT). The DNA could be stored indefinitely at -20°C or pelleted 10 min at 11,600 x g, washed once with 80%(v/v) ethanol and dried in a Speed-VacTM AES 1000 centrifugal vacuum drier (Savant Instruments, Hicksville, NY). Nucleic acid was quantitated by measurement of the absorbance of an aliquot of the sample at 260 nm using a Unicam 8730 spectrophotometer (Mississauga, ON).

RESTRICTION ANALYSIS OF DNA

Restriction digests were performed by dilution of DNA (up to 5 µg) in dH₂O and with the addition of 1 µl RNase (10 mg/ml, heat-inactivated by boiling 20 min), 2 µl restriction endonuclease buffer (10x) in a total volume of 20 µl. Up to 2 µl of restriction endonuclease(s) were added (1-5 U/µg DNA) and the reaction allowed to incubate at the specified temperature

(usually 37°C). For restriction mapping, digestions were allowed to proceed for 2-3 hrs, while for DNA fragment isolation, the digests were incubated for exactly 1 hr. Three µl of 10x gel loading dye [50%(v/v) glycerol, 25%(w/v) ficoll, 0.25% (w/v) bromphenol blue, 0.25%(w/v) xylene cyanol FF] was added, and the mixture loaded onto an agarose gel for electrophoresis. The gels were cast in removable plastic trays (11 X 14 cm) to a thickness of 3-5 mm, using 100 ml of a 1.0-1.5%(w/v) agarose (Gibco/BRL, Burlington, ON; Seakem™ or Seaplaque™ - FMC Bioproducts, Rockland, ME) solution made up in gel running buffer. The gel buffers used were 1x Tris borate EDTA (TBE) (90 mM Tris-base, 90 mM boric acid and 2 mM EDTA) or 1x Tris-Acetate EDTA (TAE) (40 mM Tris-base, 20 mM sodium acetate, 0.2 mM EDTA, adjusted to pH 7.4 with glacial acetic acid). The agarose was dissolved by microwave heating of the agarose/buffer suspension (3-4 min on 50% power), and the solution cooled to 40-50°C before 5 µl of 10 mg/ml ethidium bromide (EtBr) was added. The TBE gels were generally run at 80-120 V and the TAE gels run at 60-80 V in the appropriate 1x gel buffer (Sambrook *et al*, 1989; Ausubel *et al*, 1995).

LABELLING OF GENE PROBES TO HJ AND WEE

For the screening of the HJ library, the HJ-3' and WEEPRO oligonucleotides were labelled using the E-link kit (Gibco/BRL, Burlington, ON) following the manufacturer's instructions. The oligonucleotide probes were also 5' end-labelled using γ -[³²P]ATP (Amersham, Oakville, ON) and polynucleotide kinase as described in Sambrook *et al* (1989). For WEE library screening, DNA fragments were generated using standard PCR reaction conditions (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM MgCl₂, 200 µM of each dNTP, 2.5 U Amplitaq™, 1.0 µM of each primer) as described by Perkin Elmer (Foster City, CA). Optionally, 1 U of Perfect Match™ (Stratagene, La Jolla, CA) could be added to the reaction mixture. Fifty µl reactions were overlaid with mineral oil or with PCR Gems™ in 500 µl microfuge tubes. The reaction program consisted of 25 cycles of 1 min at 94°C, 2 min at 37°C and 3 min at 72°C, followed by a final 7 min extension at 72°C in a Perkin Elmer DNA thermocycler (Foster City, CA). The primer pairs used to generate PCR fragments in the 26S region of WEE were WEEPRO/WEEP0, WEEP5/WEEN5, WEEP7/WEEN7 and WEE3/WEEN0 (see Table 1). The reaction mix was loaded onto a 1%(w/v) TAE agarose gel containing 0.5 µg/ml EtBr.

DEAE 81 paper (Whatman, Hillsboro, OR) was used to trap the band of interest by placing an incision ahead of the DNA band of interest. A strip of DEAE 81 paper was inserted into the slot and electrophoresis continued for 15-30 min. The DEAE 81 paper containing the trapped DNA was then washed with low-salt wash buffer (10 mM Tris-HCl, pH 8.0, 0.10 M NaCl, 1 mM EDTA), followed by elution of the DNA with 50-100 μ l of elution buffer (10 mM Tris-HCl, pH 8.0, 1.5 M NaCl, 1 mM EDTA). The eluant was phenol/ CHCl_3 extracted, followed by ethanol precipitation (Danner, 1982; Sambrook *et al*, 1989). Alternatively, the fragment was isolated from low melting agarose gels by excision of the fragment, and subsequent phenol extraction of the melted agarose (Sambrook *et al*, 1989; Ausubel *et al*, 1995).

After washing and drying, the PCR fragment was labelled using the MultiprimeTM random primer labelling kit (Amersham, Oakville, ON). Briefly, 25 ng of DNA in 10 μ l of dH_2O was boiled 2 min and quickly cooled on ice. The following items were added: 11 μ l dH_2O ; 4 μ l of each of dATP, dGTP and TTP; 5 μ l buffer concentrate; 5 μ l primer/BSA; 5 μ l α [³²P]-dCTP in tricene (3,000 Ci/mmol, 50 μ Ci - Amersham, Oakville, ON); and 2 μ l of DNA polymerase I Klenow fragment (1U/ μ l). The reaction was incubated 30-60 min at 37°C, after which 50 μ l of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was added. The free nucleotides removed by passing the sample through a 1.0 ml G-50 SephadexTM (Pharmacia, Baie d'Urfé, PQ) spun-column as described (Sambrook *et al*, 1989) or through the use of NucletrapTM Push Columns (Stratagene, La Jolla, CA) following the manufacturer's instructions.

PREPARATION OF COLONY AND DOT BLOTS

Colony hybridization was based on the original procedure of Grunstein and Hogness (1975) as described in Sambrook *et al* (1989) to liberate the DNA from bacterial colonies and bind it to nitrocellulose filters (Hybond-C Extra - Amersham, Oakville, ON). Pieces of Whatman 3MM paper were cut to an appropriate size and shape to fit into the bottoms of three glass trays. The filter paper was saturated with one of the following solutions: 10% SDS; denaturing solution (0.5 N NaOH, 1.5 M NaCl); or neutralizing solution (1.5 M NaCl, 0.5 M Tris Cl, pH 7.4). Bacteria to be screened were plated out onto appropriate media (LB agar with 50-100 μ g/ml ampicillin) and grown overnight (O/N). The nitrocellulose filters were placed on top of the 1-

2 mm colonies in the 90 or 150 mm plates and left 30-60 sec. The position of the filter relative to the plate was indicated by using a 18 or 20 g needle to puncture holes through the filter and into the plate. These holes could then be used to re-align the plate to an autoradiograph of the filter after hybridization, allowing positive colonies to be selected from the plate. The filters were lifted carefully from the plate and placed colony side up on the SDS impregnated 3MM paper. After the first filter had been exposed to the SDS solution for 5 minutes, it was transferred to the second sheet of 3MM paper, saturated with denaturing solution for 5 minutes. The filters were then transferred to the third sheet of 3MM paper, saturated with neutralizing solution, and left for 5 minutes. They were then placed on a clean sheet of Whatman 3MM paper (colony side up) and the surface of the filter was blotted once with a second sheet of 3MM paper. The filters were transferred to a tray containing 2x SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0) and gently agitated for 5 min. They were dried at RT for at least 30 minutes, sandwiched between two sheets of 3MM paper and clamped between glass plates. They were baked for 1-2 hours at 80°C in a vacuum oven. Any filters not used immediately in hybridization reactions were sealed in plastic and stored at -70°C.

Dot blots were prepared for more accurate screening of recombinant plasmids with labelled probes (Kafatos *et al*, 1979). Nylon membranes (Hybond-NTM - Amersham, Oakville, ON) were spotted with 1-2 µl of plasmid preparations along with appropriate controls. After air drying, the membranes were covered with plastic wrap and placed face down on a Fotodyne Model 3-3500 UV transilluminator (New Berlin, WI) for 5 min on the long UV setting. The membranes were denatured similarly as for colony filters, using 3 min in denaturing solution, 3 min in neutralizing solution and equilibrated for 5 min in 2x SSC (Grunstein and Hogness, 1975; Sambrook *et al*, 1989).

HYBRIDIZATION TO LABELLED DNA PROBES

Hybridization and washing with the HJ3' and WEEPRO E-link oligonucleotide probes followed the manufacturer's directions. For [³²P]-labelled probe screening, nitrocellulose filters and nylon membranes were hybridized utilizing formamide solutions (Denhardt, 1966, McConaughy *et al*, 1969). The membranes were prehybridized for 1 hr at 42°C in sealed

plastic bags containing 10-20 ml Hood buffer [50%(v/v) formamide (deionized); 20 mM sodium phosphate buffer (pH 6.7); 5x SSC (0.75 M sodium chloride, 0.075 M sodium citrate, pH 7.0); 7%(w/v) SDS; 1 %(w/v) PEG MW 20,000; and 0.5%(w/v) nonfat powdered milk (Carnation)]. For large numbers of filters, teflon mesh was used to separate the layers of filters. Hybridization was carried out in 5-10 ml Hood buffer containing 10^7 cpm of radiolabelled probe (Sambrook *et al*, 1989). After hybridization, filters or membranes were transferred to polyethylene trays and washed with two quick changes of 2x SSC, 1%(w/v) SDS. They were then washed with 2 changes of 2x SSC, 1%(w/v) SDS at 42-60°C, 20 min each. A final wash of 20 min at RT in 0.1x SSC, 1%(w/v) SDS with gentle agitation was performed, and the filters or membranes were sealed in plastic for exposure with Kodak XAR-5 X-ray film (Rochester, NY).

PREPARATION OF PLASMID DNA

A scaled-up version of the Holmes and Quigley (1981) procedure for rapid preparation of bacterial plasmids was followed. Clones identified by colony hybridization were cultured O/N in 2-5 ml of LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1.0 liter dH₂O) with 50-100 µg/ml ampicillin. The cells were pelleted for 30 min at 3,000 RPM (Beckman TJ-6 bench top centrifuge - Mississauga, ON) and the supernatant discarded. The cell pellet was resuspended in 200 µl of STET buffer [8%(w/v) sucrose, 5% triton X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0] and the suspension transferred to a 1.5 ml microfuge tube. Twenty-five µl of freshly prepared lysozyme (10 mg/ml) was added and the samples boiled 1 min. The tubes were then spun for 15 min at 11,600 x g in a microfuge at RT. The supernatant was transferred to a clean 1.5 ml microfuge tube, phenol-chloroform extracted, and precipitated with an equal volume of isopropanol (Sambrook *et al*, 1989). The tube was placed at -20°C for a minimum of 30 min, and then pelleted at 11,600 x g. The pellet was washed, dried and dissolved in 100-200 µl sterile dH₂O.

Large batches of *E. coli* plasmid were isolated using the Qiagen kit (Chatsworth, CA), a method based on the alkali lysis method of Birnboim and Doly (1979), following the manufacturer's instructions. A second method used for large-scale plasmid preparation was by a procedure

involving lysis with triton X-100 (Davis et al, 1980). Cells were grown O/N in LB broth containing 50-100 µg/ml ampicillin (0.5-1.0 liter), then pelleted for 10 min at 10,000 x g (Beckman JA-14 rotor - Mississauga, Ont.). The pellet was washed in TES (50 mM Tris-HCl, pH8.0, 50 mM NaCl and 5 mM EDTA) and then resuspended in 1.5 ml of cold sucrose buffer (25% sucrose, 50 mM Tris-HCl, pH8.0 and 1 mM EDTA). After 10 min on ice, 0.15 ml of 10 mg/ml lysozyme (fresh, in dH₂O) was added and the cells were left for an additional 10 min on ice. Next, 0.6 ml of 0.25 M EDTA was added, and the cells were incubated for a further 10 min on ice. Slowly, 5.25 ml of triton lysis buffer [2%(v/v) triton X-100, 50 mM Tris-HCl, pH 8.0 and 60 mM EDTA] was added with gentle stirring, and the mixture left on ice for 15-20 min. Cell debris was removed by centrifugation for 25 min at 37,000 x g (Beckman J2-21, JA20 rotor - Mississauga, ON) after which the volume of the supernatant was adjusted to 9 ml with TE buffer. Nine grams of molecular biology grade CsCl (Gibco/BRL, Burlington, ON) was dissolved in each sample followed by the addition of 0.1 ml EtBr (10 mg/ml in dimethyl sulfoxide). The solution was transferred to Beckman heat-seal tubes, filled with paraffin oil, sealed and centrifuged for 18-24 hrs at 247,000 x g (Beckman L8-70, 70.1 Ti rotor - Mississauga, ON). The plasmid band was collected by puncture of the tube using a 18 g needle and 3 ml syringe was used to withdraw the band. The DNA was extracted with dH₂O-saturated N-butanol (Sambrook *et al*, 1989) and dialyzed against TE buffer.

DNA SEQUENCE ANALYSIS

Manual sequencing was performed using the SequenaseTM ver 2.0 reaction kit (Amersham, Oakville, ON). The plasmid template (i.e. pcDW-6, pcDW-12, XH-7) was previously purified using one of the large-scale plasmid purification methods. A 1 µg/µl plasmid stock in dH₂O was required. Five µg of plasmid template was denatured by the addition of 1/10 volume 2 N NaOH and incubated 30 min at 37°C. This was followed by neutralization with 1/10 volume of 3 M sodium acetate. The sample was then precipitated by the addition of 4 volumes of 95%(v/v) ethanol, left O/N at -20°C, pelleted, washed and resuspended in dH₂O. For each sequencing reaction, 5 µg of denatured template in 7 µl dH₂O was mixed with 1 µl of primer (0.5 pmol) and 2 µl of SequenaseTM reaction buffer. The mixture was incubated for 2 min at 65°C, then slow cooled in a beaker of water to around 30°C. One µl of 1.0 M dithiothreitol

(DTT), 2 μ l labelling mix (diluted 1/5) and 0.5 μ l α [³⁵S]dATP in tricene buffer, >1000 Ci/mmol (10 μ Ci/ μ l - Amersham, Oakville, ON) were added to the primer/template mixture. Aliquots of the primer/template mixture were added (3.5 μ l) to each of four tubes containing 2.5 μ l of the appropriate dideoxy-terminator mix (ddGTP, ddATP, ddTTP or ddCTP) which had been previously prewarmed at 37°C. The reactions were incubated 3-5 min at 37°C after which 4 μ l of dye-stop solution was added to each tube. Reaction tubes were heated 2 min at 80°C immediately prior to loading on the gel. After the wells were flushed, 2-3 μ l of each reaction mix was loaded in sets of four dideoxy-terminators (GATC) using flat Gel-Loader pipette tips (Gordon Technologies, Mississauga, ON; National Scientific, Claremont, CA) and a Gilson PipettemanTM P-20 pipettor (Mandel Scientific, Guelph, ON). After a 2-4 hr run time, a second set of the samples could be loaded into any remaining wells. Forty well sharktooth combs were used for 40 cm gels. A number of different gel formats were run, but generally consisted of 8% acrylamide (1:20 bis-acrylamide:acrylamide ratio), 8 M urea and 1x TBE buffer. Forty cm and 90 cm, 0.4 mm thick gels using Gibco/BRL (Burlington, ON) S0 and S2 sequencing units, respectively, were run at 40-50 W constant power on BioRad (Mississauga, ON) 3000 power supplies. When the bromphenol blue dye was near the bottom, the gel was removed, fixed with three changes of 10%(v/v) methanol/10%(v/v) acetic acid (5 min per change). The gel was rinsed with several changes of dH₂O, followed by transfer to a sheet of Whatman 2 or 3 MM filter paper. Ninety cm gels were cut into two equal pieces prior to transfer to the Whatman filter paper. The gel was covered with plastic wrap, and dried on a gel drier under vacuum for 30 min at 80°C. The plastic wrap was removed, and the dried gel was exposed to Kodak (Rochester, NY) XAR-5 X-ray film (14" x 17") with a Dupont CronexTM intensifying screen (Mississauga, ON). The X-ray was developed in Kodak (Rochester, NY) GBX developer and fixer and analysed on a Speed ReaderTM semi-automated DNA sequence imager (Intelligenetics, Mountain View, CA). The edited sequence files were transferred to text format using PC/GeneTM (Intelligenetics, Mountain View, CA) DNA analysis software, prior to transfer to the LasergeneTM biocomputing software (DNASTAR Inc., Madison, WI) installed on a Power MacintoshTM 8100/80 (Apple Computer, Cupertino, CA) for sequence assembly.

Automated sequencing was performed using an Applied Biosystem Instruments (ABI) 373 automated fluorescent sequencer and the ABI PrismTM Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA). Both AmplitaqTM and Amplitaq FSTM were used on Qiagen purified dsDNA plasmid templates according to the manufacturer's instructions. The reactions were run on a Perkin Elmer thermocycler (Foster City, CA) using 30 cycles of: 96°C for 30 sec; 45°C for 30 sec and 60°C for 4 min. Reactions were purified on Centri-SepTM columns (Princeton Separations, Adelphia, NJ), dried and run on an ABI 373 automated sequencer (USAMRIID, Regional DNA Synthesis Laboratory). Chromatographic data obtained was downloaded onto the MacIntosh 8100/80 and edited in SeqEdTM ver 1.0.3 (ABI, Foster City, CA), or transferred directly to LasergeneTM (DNASar, Madison, WI) for sequence assembly. ThermosequenceTM reactions were also compared to the Amplitaq FSTM according to the manufacturer's protocol (Amersham, Oakville, ON), however, the results were no better than the Amplitaq FSTM (Perkin Elmer, Foster City, CA).

CONSTRUCTION OF THE FULL-LENGTH 26S REGION OF WEE

A clone, pcDW-12, containing a 3100 bp fragment of the 26S region of WEE strain 71V-1658 was identified from the WEE library by hybridization to WEE probes, restriction mapping and DNA sequencing. The 5' end of the 26S region was synthesized from the WEE 71V-1658 RNA using standard PCR reaction conditions, and the WEE5'Sst and WEEP3 primers as outlined in Fig. 1. Before the PCR reaction was run, a reverse transcription (RT) of the RNA template was completed in the same tube using the PCR reaction conditions by programming in a 42°C "soak" cycle before the start of the thermocycling reaction. The changes incorporated were as follows: the primers were allowed to slow anneal to the RNA template, followed by the addition of 18 U of AMV reverse transcriptase (Life Sciences, Petersburg, FL) and 2.5 U AmplitaqTM polymerase (Perkin Elmer, Foster City, CA). The reaction was incubated for 30-60 min at 42°C, and then the standard PCR cycling was programmed to run. The PCR products were then analyzed on agarose gels. An 1800 bp fragment of the estimated size (Hahn *et al*, 1988) was isolated as previously described. The fragment was then restriction digested with *NcoI* and *SstI*, gel purified, and ligated into the *SstI/NcoI* sites of plasmid, pH3T7BM+ (Boehringer Mannheim, Laval, PQ). To expand on the details, the *SstI/NcoI* digested

phT3T7BM+ (gel purified, 50-100 ng) was added to the *SstI/NcoI* 1800 bp PCR fragment generated from WEE5'Sst/WEPP3 primers (1:3, 1:1 and 3:1 molar ratios) in a total volume of 15 μ l dH₂O. Controls with digested plasmid DNA only and insert DNA only were also included. The DNA was slowly annealed from 65°C, followed by the addition of 4 μ l of 5x ligase buffer (Gibco/BRL, Burlington, ON) (250 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 25%w/v PEG 8000, 5 mM ATP, 5 mM DTT) and 1 U T4 DNA ligase (Gibco/BRL, Burlington, ON; Pharmacia, Baie d'Urfé, PQ). The reaction was incubated for 4 hr at 15°C in a refrigerated waterbath. Fifty μ l of TE buffer was added and 2-5 μ l of diluted ligation mixture was used to transform competent *E. coli* cells.

The transformation protocol of Hanahan (1983) was followed. Competent DH5 α (maximum efficiency) *E. coli* cells were used, and purchased from Gibco/BRL (Burlington, ON). Briefly, 2-5 μ l of diluted ligation reaction was added to 100 μ l of cells in pre-chilled 15 ml snap cap tubes. The tubes were left for 30 min on ice, heat shocked for 45 sec at 42°C and then cooled 2-3 min on ice. One ml of 2x YT [1 %(w/v) tryptone, 1 %(w/v) yeast extract, 0.5 %(w/v) NaCl] or SOB [2 %(w/v) tryptone, 0.5 %(w/v) yeast extract, 8.2 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄] broth was added and the tubes incubated at 37°C for 30 min with gentle agitation. The cell suspension (100 μ l) was plated out onto a LB plate containing 50-100 μ g/ml ampicillin.

A recombinant plasmid containing the 1800 bp WEE 5' end (phTW5'-3) was identified by restriction analysis of rapid plasmid preparations and confirmed by fine restriction mapping and DNA sequence analysis. The *XbaI/NcoI* fragment of phTW5'-3 (WEE 5' 1800 bp recombinant in phT3T7BM+) was ligated into the *XbaI/NcoI* sites of pcDW-12. After transformation into DH5 α , a full-length recombinant containing the complete WEE 71V-1658 26S region was identified (XH-7) for further analysis. Additional cDNA constructs to WEE strain 71V-1658 were made using primers WEE5'Sst and WEE3'HindIII (see Table 1) for the RT-PCR amplification and directional cloning of the full-length WEE 26S into Invitrogen vector pRSETA (cloned into the *SstI* and *HindIII* sites).

PCR AMPLIFICATION OF THE NONSTRUCTURAL REGION OF WEE

A PCR cloning strategy was employed to produce a set of three overlapping fragments (WNST-A, B and C) for the nonstructural region of WEE71V-1658 (Fig. 2). RT-PCR reactions were carried out using three sets of PCR primers as listed in Fig. 2. For WNS-A fragment, two different combinations of primers were made, using a SIN5' or EHJ5' upstream primer, respectively. RT-PCR amplified cDNA was gel purified, and ligated into the modified EcoRI site of the pCRII vector supplied with the TA-cloning systemTM (Invitrogen, San Diego, CA). The recombinant plasmid was transformed into *E. coli InvaF'* competent cells as described (Invitrogen, San Diego, CA). Isolates were screened by restriction mapping rapid plasmid preparations and by DNA sequencing using the T7 and M13 reverse primers (Invitrogen, San Diego, CA).

RESULTS

HJ AND WEE LIBRARY SCREENING

Plaque assays of the batches of WEE 71V-1658 grown at USAMRIID were completed (data not shown). The titre of the 10% SMB of WEE 71V-1658 was 6.1×10^8 pfu/ml. The seed stock was 4.1×10^7 pfu/ml. The quality of cDNA libraries is dependent foremost, on the integrity of the RNA starting material. RNA gels of HJ virus strain B320 and the initial batch of WEE 71V-1658 (CDC) indicated some degradation in the samples had occurred (data not shown). This is reflected in the large numbers of small inserts (<1000 bp) encountered when screening isolates of HJ library for insert size (see Fig. 3 and Table 2). This was surprising since Invitrogen indicated that the cDNA synthesis had attained an excellent size distribution, ranging from 100 bp to >8,000 bp. In addition, the cDNA was size selected (>2,000 bp) prior to ligation and transformation into the pcDNA II vector. In any event, we used oligonucleotide probes to the HJ 3' end (Ou *et al*, 1981) and the promoter region (WEEPRO primer) of WEE (see Table 1). As genomic sequence information for HJ was limited to the 3' and 5' ends, we had speculated that if HJ and WEE were related, the 26S promoter area could show conservation of the genome sequence and cross-reactivity to HJ. A new, nonradioactive

procedure (E-link) was used to label HJ3' and WEEPRO oligonucleotide probes to screen isolates of interest (26S region). However, better hybridization results were achieved using probes conventionally labelled at the 5' end of the oligonucleotide with γ -[^{32}P]ATP and polynucleotide kinase. Nevertheless, over 100 isolates were screened by restriction mapping (see Fig. 3) and oligonucleotide probe reactivity (see Table 2). The size distribution of the inserts was as follows: 0-500 bp - 32%; 500-1000 bp - 35%; 1000-2000 bp - 26%; and >2000 bp - 7%. Non-reactivity with the probes would only indicate that the isolate did not carry an insert that extended into the limited target region of the probe, and could not be interpreted as the isolate carrying no HJ DNA. A number of HJ clones were identified for DNA sequence analysis including: A19, A24, A35, B23, C12, D1 and D2). Further work was not completed with Highlands J isolates, as the emphasis shifted to the WEE strain 71V-1658 library.

As previously mentioned, the initial batch of WEE 71V-1658 showed degradation by RNA formaldehyde gel electrophoresis (data not shown), however, subsequent batches of genomic RNA of WEE 71V-1658 were very good (aliquots stored in ethanol at -70°C). Sequence data was available to the entire 26S region of WEE strain BFS 1703 (Hahn *et al*, 1988) and was used to design a set of primers that could be used for PCR amplification, as well as DNA sequence analysis of WEE strain 71V-1658 (see Table 1). The fragments generated by PCR were designed to overlap at unique restriction sites, and allowed PCR generated fragments to be substituted into the WEE 26S clones. This was used to our advantage in the subsequent genetic engineering of a full-length 26S clone, as a complete clone could not be isolated from the Invitrogen library. Rapid plasmid isolates were screened by dot blot analysis using WEEPRO/WEEPO, WEEN5/WEEP5, WEEN7/WEEP7 and WEENO/WEE3' PCR generated fragments as probes (see Fig. 4). The results from dot hybridization are summarized in Table 3. Dot blot hybridization was used to quickly size inserts in the WEE library. The PCR probes selected were spaced along the 26S gene segment. We theorized that a full-length 26S insert would hybridize with all four PCR probes, while smaller the inserts would only hybridize to one or two probes. No isolates were identified which hybridized to all four probes, but a number of the largest isolates hybridized to three of the four probes. These included pcDW-1, 4, 6, 7 and 12 (data not shown). The size distribution of inserts in the pcDNA II vector was similar to that obtained with the HJ library, with not many large inserts obtained (>2,000 bp).

The largest clones identified were pcDW-6 and pcDW-12, which were estimated to be 2900 and 3100 bp, respectively, by restriction analysis on agarose gels (see Table 3). This was still 25-30 % shorter than the estimated 4100-4200 size of the 26S region of WEE. DNA sequence analysis confirmed that both clones pcDW-6 and pcDW-12 were truncated from the 5' end of the WEE 26S region at nucleotide (nt) 1798 and nt 1446 (BFS 1703 numbering system - Hahn *et al*, 1988), respectively. However, since the WEE sequencing primers were designed to produce fragments which terminated at unique restriction sites (BFS 1703 WEE 26S sequence - Hahn *et al*, 1988), a strategy was developed to place a PCR synthesized 5' fragment onto the end of pcDW-12 as outlined in Fig. 1.

CONSTRUCTION OF THE FULL-LENGTH 26S REGION OF WEE

RT-PCR was used to synthesize the WEE 26S 5' end using the WEE5'Sst and the WEEP3 primers. A fragment of the expected size (1800 bp) was isolated from agarose gels and ligated into the *Sst*I and *Nco*I sites of phT3T7BM+ (data not shown). A number of recombinant plasmids containing the 1800 bp WEE 5' end were screened by restriction analysis of rapid plasmid preparations, and confirmed by fine restriction mapping and DNA sequence analysis (phTW5'-3, 4 and 6 - data not shown). Cloning into phT3T7BM+ allowed us to excise the same 1800 bp fragment with the addition of a *Xba*I site at the 5' end. The *Xba*I/*Nco*I fragment of phTW5'-3 was ligated into the *Xba*I/*Nco*I sites of pcDW-12 and full-length 26S region clones (XH-7, 12) were screened for by restriction analysis of rapid plasmid preparations (Fig. 5). XH-7 was fine mapped (data not shown) and demonstrated an almost identical restriction fragment pattern to WEE strain BFS 1703 (Hahn *et al*, 1988).

DNA SEQUENCE ANALYSIS OF THE 26S REGION OF WEE

XH-7 was completely DNA sequenced using a combination of manual (see Fig. 6) and automated sequencing (see Fig. 7). The manual sequencing was very time consuming when compared with the ABI (Foster City, CA) automated sequencer. Manual sequences were analysed on the Speed ReaderTM (Intelligenetics, Mountain View, CA). While being an improvement over the visual reading of autoradiographs of DNA sequencing gels, it is still a

slow and tedious process as compared to automated fluorescent systems. A complete DNA sequence was obtained for clone XH-7 (see Appendix A) using the primers listed in Table 1. In the nucleotide sequence, 63 nt changes were detected, with only 11 causing amino acid substitutions from WEE BFS 1703 (see Fig. 8). The 63 nt changes were generally scattered throughout the 26S gene segment as follows: 5' noncoding region - 1; NC - 16; E3 - 2; E2 - 15; 6K - 1; E1 - 19, 3' noncoding region - 9. The 26S promoter (101-124) and the tandem repeats (3896-3935, 3954-3993) in the 3' noncoding region were conserved (1 nt change to make it an exact repeat in 71V-1658 - Appendix A). The XH-7 construct had a continuous open reading frame beginning and terminating at the same amino acid residues as strain BFS 1703 (Appendix B). The amino acid sequence was highly conserved, with 10 of the 11 amino acid differences being conservative changes. Only the Cys to Arg (28) in the NC protein would be considered nonconserved (see Fig. 8). The XH-7 clone was truncated at the 3' end of the genome with respect to the BFS 1703 sequence. This is likely a deletion, as restriction analysis of the pcDW-12 as compared with pcDW-6 and 7, showed a smaller band mapping to the 3' end (1600 bp instead of 1700 bp for pcDW-6 and 7). In addition, the poly A tract at the 3' end was completely missing. To confirm this, the 3' ends of both pcDW-6 and pcDW-7 could be sequenced. Further full-length cDNA constructs to WEE strain 71V-1658 were made using primers WEE5'Sst and WEE3'HindIII in a RT-PCR reaction. A set of XTRAH clones were isolated, and XTRAH-5 was characterized by restriction mapping (data not shown) as containing the entire 26S coding sequence.

ANALYSIS OF THE 5' WEE NONSTRUCTURAL REGION

One of the approaches initially examined for vaccine research was to obtain an infectious clone for mutagenesis studies. In order to accomplish this task, the entire genome of WEE must be cloned. Sequence information was available to the middle of the nonstructural region (Weaver *et al*, 1993), however, no sequence information was available for the 5' end of the nonstructural region. Nevertheless, it was deduced that the 5' termini from a number of alphaviruses were highly conserved (Ou *et al*, 1983), and this information was used to devise a cloning strategy for the region (see Fig. 2). Two primers were made to the 5' termini, one based on the SIN sequence (SIN5') and the other based on a combination of the EEE and HJ sequences (EHJ5').

RT-PCR reactions were carried out using three sets of PCR primers as indicated in Fig. 2, to generate 3 overlapping fragments for reconstruction of the WEE nonstructural region. For amplification of the 5' nonstructural gene fragment (WNS-A), the EHJ5' primer worked well (see Fig. 9), generating a band of the expected MW. This is in contrast to the SIN5' primer which generated only a multitude of smaller sized bands, of which many were eliminated when Perfect MatchTM was added to the reaction mix (see Fig. 9). In general, Perfect MatchTM increased the stringency of the PCR reaction, eliminating some extraneous bands. Following gel purification and ligation of the WNS-A, WNS-B and WNS-C fragments, transformant colonies were identified by colony hybridization, and rapid plasmid preparations were restriction analyzed (data not shown). Isolates of the proper size were identified for WNS-A (WNSTA-3, 4, 5 & 6) and WNS-C (WNSTC-1, 2, 3 & 4) fragments. These were sequenced with the T7 and M13 reverse primers from the pCRII multicloning region.

The clustal method of homology comparison was used for both DNA and protein sequences in the LasergeneTM program. The WNSTA-3 5' DNA sequence showed high homology to HJ (Ou *et al*, 1983), EEE North American (NA) (Weaver *et al*, 1994) and EEE South American (SA) (Ou *et al*, 1983) as illustrated in Fig. 10a and Appendix C. Homology to SIN strain HR was more distant (data not shown), as illustrated in the phylogenetic tree (see Fig. 10b). The First 19 nt of WNSTA-3 are derived from the EHJ5' primer and were not included in the homology calculations, as they may not correspond exactly to the WEE 71V-1658 5' end of the genome. If the corresponding, translated open reading frames of WNSTA-3, HJ, EEE-NA, EEE-SA and SIN are compared, fairly high homology is obtained for WEE, HJ, EEE-NA and EEE-SA, as shown in Fig. 11a. Structures which were highly conserved in all alphaviruses, such as a 51 nt sequence (nt138-188) and a 22 amino acid sequence (residues 34-55) (Ou *et al*, 1983) were also present in the WEE sequence. The 3' end of WNSTA-3 was sequenced for 233 nt, and a comparison to the BFS-1703 sequence (Weaver *et al*, 1994) using the Clustal method yielded very high homology.

DISCUSSION

When vaccine research on WEE was formulated at the start of the project, Highlands J strain B320 was included, as its crossreactivity and reduced virulence in humans made it a good vaccine candidate. Previous studies had postulated that HJ was a variant of WEE based on its antigenic cross-reactivity (Hayes and Wallis, 1977; Karabatsos *et al*, 1988) and it was referred to as the eastern strain of WEE virus in the early literature. A cDNA library to HJ strain B320 was produced containing a number of isolates whose inserts were reactive with the HJ probe (see Table 2). However, with the limited sequence information available and the priority to produce a WEE specific subunit vaccine, the research focus was shifted to WEE. Nevertheless, in order to glean further information on the pathogenesis of WEE, it would be of interest to define how HJ virus is related, through DNA sequence analysis.

The WEE library was disappointing in that a full-length clone of the 26S region could not be obtained. The largest clones were estimated to be 2900 and 3100 bp respectively, for pcDW-6 and pcDW-12. The longer clone, pcDW-12 was chosen to serve as the starting material for engineering of the full-length 26S region (Fig. 1). Utilizing the advances in PCR cloning, the 5' end of phTW5'-3 was fused at the *NcoI* site to the bulk of pcDW-12 to form the XH-7 clone. A set of monoclonal antibodies to WEE strain B11 have been made and partially characterized by ELISA and western blot analysis (unpublished results). These were reactive to inactivated WEE 71V-1658 in western blots, and will be valuable in defining levels of protein produced *in vitro* (tissue culture, baculovirus, vaccinia) or *in vivo* (DNA immunization). The restriction map of 71V-1658 was highly conserved when compared to BFS 1703 and it could be used to restriction map isolates and directional clone (cloning using two different restriction sites) PCR fragments.

One technological change which had an immense impact on the outcome of the project was the switch in DNA sequencing from a manual method to an automated fluorescent method. The amount of sequence data that can be generated by the fluorescent sequencers is astounding. A year's worth of work could be easily cut down to one or two months. The need for radioisotope was eliminated and samples were easier to set up, run and analyze. The raw sequence data

(ABI chromatograms) could be directly imported into the Power Macintosh-based LasergeneTM software for evaluation.

The WEE virus strain 71V-1658 was selected by Dr Trent as a vaccine isolate, as it was a relatively recent isolate of WEE. The surprisingly high degree of homology between the two strains of WEE is extended to the nucleotide sequence, as shown by homology comparison in DNASTAR to the BFS 1703 sequence (see appendix A). The majority of the 63 nt changes were silent mutations. Out of eleven amino acid changes in the polyprotein, 10 produced conservative amino acid changes, and only one at residue 28 (Cys → Arg) could be considered radical. The viruses were isolated around the same time period, however, this high degree of conservation was also noted with a South American isolate, WEE strain CBA (personal communication - M. Parker, USAMRIID). In addition, the N-terminal regions of the E1 and E2 proteins were also conserved when compared to the McMillan strain of WEE which was isolated in 1957 (Bell *et al*, 1983). From over 60 residues which were determined for the N-termini of each of E1 and E2 proteins of the McMillan strain, only 3 amino acid changes were noted for 71V-1658 (see Fig. 8). With these indications that the amino acid sequence is highly conserved amongst WEE strains, one strain of WEE should be able to provide protection to most of the WEE isolates. While the 3' noncoding region of XH-7 was deleted (21 nt), it should not affect the expression of the structural genes, especially in constructs which contain a poly-A tailing and/or a transcription terminator signal.

The 5' end of the WEE genome was surprisingly well conserved when compared with the 5' end of the HJ or EEE genomes (see Fig. 10a). It was previously hypothesized that WEE was a recombinant virus, derived from EEE and a SIN -like virus, with the nonstructural region and capsid protein derived from EEE (Hahn *et al*, 1988). This data confirms that the 5' end of WEE is derived from EEE and not from a SIN ancestor (see Fig. 10b and 11b). The limited HJ virus sequence was also highly conserved in this region of the genome, however, more sequence data for HJ will have to be obtained to draw definitive conclusions. The 2.2 kb WNS-A fragment represents a part of the WEE genome that has not been examined to date. In the course of the research, an infectious clone approach had been dropped in favor of a subunit vaccine approach

(DNA immunization, baculovirus or yeast expression). However, it would be of interest to complete the sequencing of the WNS-A fragment from a scientific point of view.

CONCLUSION

A full-length clone for the 26S region of WEE strain 71V-1658 has been obtained and completely sequenced. Only eleven of the sixty-three nucleotide changes resulted in amino acid changes when the sequence was compared to WEE strain BFS-1703. The high degree of conservation of the structural proteins was maintained when compared to the N-terminal regions of the E1 and E2 envelope proteins of the WEE McMillan strain. The conserved nature of the structural proteins would indicate that one strain of WEE should be able to cross-protect against all WEE strains. Furthermore, the 5' end of the genome was cloned as a 2.2 kb fragment and sequenced for 247 nt on 5' end and 233 nt on 3' end of the fragment (WNS-A). It demonstrated high homology to both HJ and EEE, adding further evidence that the entire 5' nonstructural region of WEE was derived from EEE. This data also demonstrates the close relationship between HJ and WEE at the genetic level, which was previously implied by antigenic cross-reactivity. In summary, the genetic cloning and sequencing of the WEE 26S region marks the critical first step in the generation of a subunit vaccine to WEE.

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Table 1: WEE 26S REGION PRIMERS

✓ • Name	Length	Sequence	Note
✓ HJ-3'	30	GCATTATGCACTGTGCTTCCTCGGAAGTAC	Highlands J 3' end
✓ WEEPRO	30	AATCACCCCTCTACGGCTGACCTAAATAGGT	WEE 26S 96-125, + STRAND PROMOTOR
✓ WEEPR-SST	24	GGCTGAGCTCAATAGGTGACGTAG	WEE 26S 109-132, + STRAND SstI SITE
✓ • WEE3'	30	GTAGTGTATATTAGAGACCCATAGTGAGTC	WEE 26S 4086-4057, - STRAND
✓ WEE5'Sst	20	TCCAGATACGAGCTCATACT	WEE 26S 1-20, + STRAND SstI SITE
✓ WEEN1	20	GGTGCCGCTGGAGGCCGTTT	WEE 26S 238-257, + STRAND
✓ WEEN1A	20	GATCTTAGGAGGTCGATAGC	WEE 26S 285-304, + STRAND
✓ • WEEN2	20	GGCTGATGAAACCACTCCAC	WEE 26S 547-566, + STRAND
✓ • WEEN3	20	CCACCCGTGTGCTATTCACT	WEE 26S 990-1009, + STRAND
✓ • WEEN3A	20	CGCCGTGTTTCAGCCCAATA	WEE 26S 990-1009, + STRAND
✓ • WEEN4	20	TCACGAGCGGAGCATCTGAG	WEE 26S 1459-1478, + STRAND
✓ • WEEN5	20	GGCATCACCCCTCCACCTGAC	WEE 26S 1959-1978, + STRAND
✓ • WEEN6	20	TTGTTATTCTGTTCCGCTGC	WEE 26S 2469-2488, + STRAND
✓ • WEEN7	20	CTATTGATCATGCAGTCGCA	WEE 26S 2893-2912, + STRAND
✓ • WEEN8	20	AGTGGAGCCTCTGCGAGCGT	WEE 26S 3335-3354, + STRAND
✓ • WEEN9	20	GAGGAGTGGGCGGGAAAGGC	WEE 26S 763-782, + STRAND
✓ • WEEN10	20	CTAAACTCGATGTATTTC	WEE 26S 3952-3971, + STRAND
✓ • WEEN11	20	ACGCGAACGAAGATGAACGG	WEE 26S 1752-1771, + STRAND
✓ • WEEN12	20	ACTGTCATTGTGCTGTGTGG	WEE 26S 2214-2233, + STRAND
✓ • WEEN13	20	CACAGTCATTCTTCACCAC	WEE 26S 2705-2724, + STRAND
✓ • WEEN14	20	CGTCATCAGAAAGGGGCTTG	WEE 26S 3080-3099, + STRAND
✓ • WEEN15	20	CAAAGCTGACAGGGAGGGAC	WEE 26S 3509-3528, + STRAND
✓ • WEEN16	20	GGAAAGCTGGTAAAGTGCCA	WEE 26S 1554-1573, + STRAND
✓ WEEN0	20	GGAGAACCACATAAAGTCGA	WEE 26S 3714-3733, + STRAND
✓ WNSP1	25	GGCTAACGTGGACAGGGACGTGATG	WEE 26S 57-33, - STRAND
✓ • WEEPO	20	GGCTATCGACCTCCTAAGAT	WEE 26S 305-286, - STRAND
✓ • WEEPOA	20	CTGTCGGTTCCTGTTTAG	WEE 26S 458-439 - STRAND
✓ • WEEP1	20	CTGGGGAACGTCGCCATACT	WEE 26S 656-637, - STRAND
✓ • WEEP2	20	CGTTCTCCAGCAGCGTGTCG	WEE 26S 1083-1064, - STRAND
✓ • WEEP2A	20	TATTGGGCTGAAACACGGCG	WEE 26S 1202-1183, - STRAND
✓ WEEP3	20	CTTCAAGTGATCGTAAACGT	WEE 26S 1592-1573, - STRAND
✓ • WEEP4	20	ACTCCAGCCCTTCTCGCCCC	WEE 26S 2097-2078, - STRAND
✓ WEEP5	20	GTTTCGACCAACGCCTTATAC	WEE 26S 2610-2591, - STRAND
✓ • WEEP6	20	AAGGGTGAAAAAGCGGCTGA	WEE 26S 3067-3048, - STRAND
✓ WEEP7	20	GGTGATTCTGATGATCTCAC	WEE 26S 3430-3411, - STRAND
✓ • WEEP8	20	TGGAAACTGCCGCCTGGAAT	WEE 26S 3759-3740, - STRAND
✓ • WEEP10	20	CCTTGATGTCATGGTCGTGG	WEE 26S 1344-1325, - STRAND
✓ • WEEP11	20	TGCACTGAGTGGTCTGTGTG	WEE 26S 1870-1851, - STRAND
✓ • WEEP12	20	ATGTTTCAGCGTTGGTTGGC	WEE 26S 2391-2372, - STRAND
✓ WEEP13	20	GTGTTCTCACTGTCACAGAA	WEE 26S 2851-2832, - STRAND
✓ • WEEP14	20	ATGTGTGGTCGCTTCCTTCA	WEE 26S 3584-3565, - STRAND

Table 2: SUMMARY OF HJ ISOLATES

Isolate	HJ-3'	WEE	Size of Inserts
A13	- - +	- - -	1400, 500, 450
A14	w - -	- - -	ND
A15	+ - -	- - -	ND
A16	w - -	w - -	500
A17	- w -	w - -	ND
A18	ww -	w - -	ND
A19	w ++	w - -	1000
A20	w - -	w - -	ND
A21	w - -	w - -	ND
A22	ww +	w - -	1200
A23	- w +	- - -	500
A24	- + +	- - -	500, 450
A25	- - -	- - -	300
A26	w - -	- - -	3K?, 500, 300
A27	ww +	- - -	600
A28	w - -	- - -	3k?, 600
A29	w - w	- - -	1400, 600
A30	- w +	- - -	1000
A31	- - -	w - -	500
A32	- - -	w - -	1400
A33	w - w	w - -	1400
A34	- w +	w - -	400
A35	ww +	+ - -	1600, 400
A36	- w +	- - -	400
B13	- - +	- - -	1000, 400
B14	- - -	- - -	450
B15	w - -	- - -	ND
B16	+ - -	- - -	ND
B17	w - w	- - -	600?, 500
B18	ww +	- - -	600
B19	+ - -	w - -	ND
B20	- w +	w - -	600
B21	w - +	w - -	500
B22	w - w	w - -	500
B23	+ w +	+ - -	550, 400

Isolate	HJ-3'	WEE	Size of Inserts
B24	w ++	+ - -	500
C1	- - -	- - -	ND
C2	- - -	- - -	1700
C3	- - -	- - -	300
C4	- - -	- - -	450
C5	w - -	w - -	650
C6	w - w	w - -	450
C7	- - w	+ - -	900, 300
C8	- w w	w - -	350, 400
C9	ww +	w - -	600
C10	ww -	+ - -	600
C11	www	+ - -	1800
C12	w ++	+ - -	850
C13	- ww	- - -	1000, 300
C14	- ww	- - -	300
C15	- ww	- - -	700
C16	www	- - -	1000?, 800
C17	www	w - -	700, 600, 500
C18	- - -	w - -	600
C19	+ - -	+ - -	3K?, 600
C20	- - +	w - -	1700, 1300, 650?
C21	- - -	- - -	650, 500
D1	w ++	+ - -	1000
D2	+ w +	+ - -	600
D3	w - -	- - -	1800
D4	- - -	- - -	400
D5	w - -	- - -	400
D6	- - w	- - -	600
D7	+ - -	w - -	550
D8	+ - -	w - -	300
D9	- - w	w - -	400
D10	+ - -	w - -	450
D11	+ - -	+ - -	1100
D12	www	+ - -	1400
D13	- - -	+ - -	ND

Isolate	HJ-3'	WEE	Size of Inserts
D14	- - -	+ - -	ND
D17	- - -	- - -	400
D18	- - -	- - -	400
D19	- - -	- - -	2K, 600
D20	- ww	- - -	1000, 400
D22	- - +	- - -	900, 400?, 300
D23	- - -	- - -	950
D24	- - -	w - -	400
D25	w - -	w - -	1200, 600?
D26	- - -	- - -	1700
D27	- - -	- - -	400
D28	- - -	- - -	650
D29	+ - -	- - -	3K?, 1500
D30	w - -	- - -	1400?, 1200
D31	- w -	- - -	1200?, 800
D32	w - -	- - -	750
D33	w - +	- - -	600, 500
D34	- - w	w - -	400
D35	- - w	w - -	400
D36	- w +	w - -	500
D37	- - -	w - -	650, ?
D38	- - -	+ - -	1300, 400?
D39	- - w	- - -	1350, 400
D40	- - -	- - -	800
D41	+ ww	- - -	800, 300
D42	ww +	- - -	800, 350
D43	w - w	- - -	400
D44	- - w	- - -	400, 300
D45	- - w	w - -	1000
D46	- - w	w - -	1400
D47	- - -	w - -	350
D48	- - w	w - -	400
D49	- ww	w - -	1000
D50	- ww	w - -	1300
D51	- ww	w - -	700, 500?

HJ-3', WEE (WEEPRO) PROBES - N-LINK, 32P-HYBOND-N
32P-HYBOND-C, RESPECTIVELY

SIZE - SIZE OF INSERT AFTER EXCISION WITH XHOI
AND HINDIII
ND - NOT DETERMINED

VG PROSPECTS - A19, A24, A35, B23, C12, D1, D2
GOOD PROSPECTS - A13, A15, A22, A30, B13, B24, C19,
C20, D11, D22, D29, D33, D41, D42

TABLE 3: SUMMARY OF WEE ISOLATES

pcDW	A	B	C	D
1	+	+	+	-
2	+	+	w	-
3	+	+	w	-
4	+	+	+	-
5	w	w	-	-
6	+	+	+	-
7	+	++	++	-
8	+	w	-	-
9	+	w	-	-
10	+	w	-	-
11	++	++	-	-
12	++	++	+	-
13	+	+	-	-
14	++	+	+	-
15	+	+	+	-
16	+	+	+	-
17	+	+	+	-
18	w	-	-	-
19	+	w	-	-
20	w	w	-	-
21	++	+	-	-
22	+	w	-	-
23	++	++	-	-
24	+	w	-	-
25	+	+	-	-
26	w	+	-	-
29	w	w	-	-
30	+	+	+	-
31	w	w	-	-
33	+	w	-	-
34	++	++	w	-
35	+	w	+	-
36	w	w	-	-
38	w	-	-	-
39	+	+	+	-

pcDW	A	B	C	D
40	+	w	w	-
41	+	+	-	-
42	+	+	-	-
43	w	w	-	-
44	+	+	-	-
45	++	+	-	-
46	+	w	-	-
47	++	+	-	-
48	+	+	-	-
49	+	+	+	-
50	w	w	w	-
53	+	+	+	-
54	+	w	-	-
55	+	w	-	-
56	w	w	-	-
57	+	w	-	-
59	+	+	w	-
61	+	w	-	-
62	+	w	-	-
63	+	w	-	-
64	w	w	-	-
67	+	w	-	-
68	w	-	-	-
69	+	w	-	-
70	w	w	-	-
71	+	w	-	-
72	w	w	-	-
73	+	+	-	-
74	+	+	++	-
75	+	w	-	-
76	w	w	-	-
77	w	w	-	-
78	+	+	-	-
79	+	w	-	-
80	-	-	-	-

pcDW	A	B	C	D
81	w	w	-	-
84	w	-	-	+
85	-	w	-	-
86	-	w	-	-
87	w	+	-	-
89	-	w	-	w
91	w	w	-	w
93	w	w	-	-
96	w	w	-	-
97	+	w	-	-
98	w	w	-	-
99	w	w	-	-
100	+	+	-	-
103	-	w	-	w
109	-	w	-	-
110	w	w	-	-
111	w	w	w	-
112	w	w	-	-
114	-	w	-	-
115	-	-	-	-
116	-	-	-	-
118	-	-	-	-
119	-	-	-	-
120	-	w	-	-
121	-	w	-	-
122	-	-	-	-
123	-	w	-	-
124	+	w	-	w
125	-	w	-	-
126	-	w	-	-
127	-	w	-	w
128	-	w	-	-

PCR Probes: A - WEEN0/WEEN3' B - WEEN7/WEEN7 C - WEEN5/WEEN5 D - WEEP0/WEEN0
 Only positive isolates included in Table.

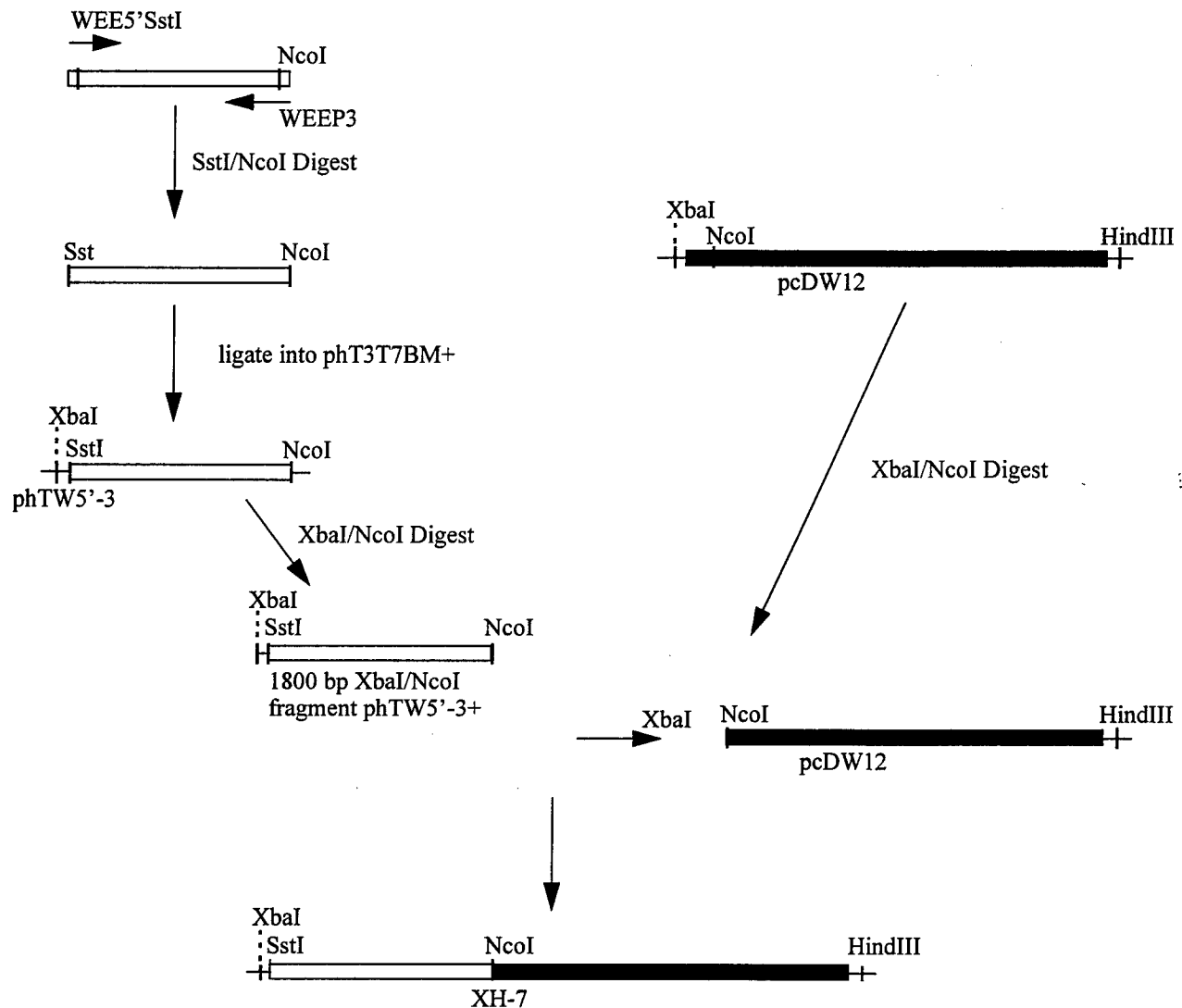


Figure 1. Construction of the Full-length WEE 26S Region

The 5' end of the 26 S region of was constructed using RT-PCR with the *WEE5'SstI*/*WEEP3* primers as described. An 1800 bp fragment was cloned into *SstI/NcoI* sites of *phT3T7BM+*. The *XbaI/NcoI* fragment (from *phTW5'-3*) was cloned into the *XbaI/NcoI* sites of *pcDW-12* to produce the full-length WEE 26S region isolate, *XH-7*.

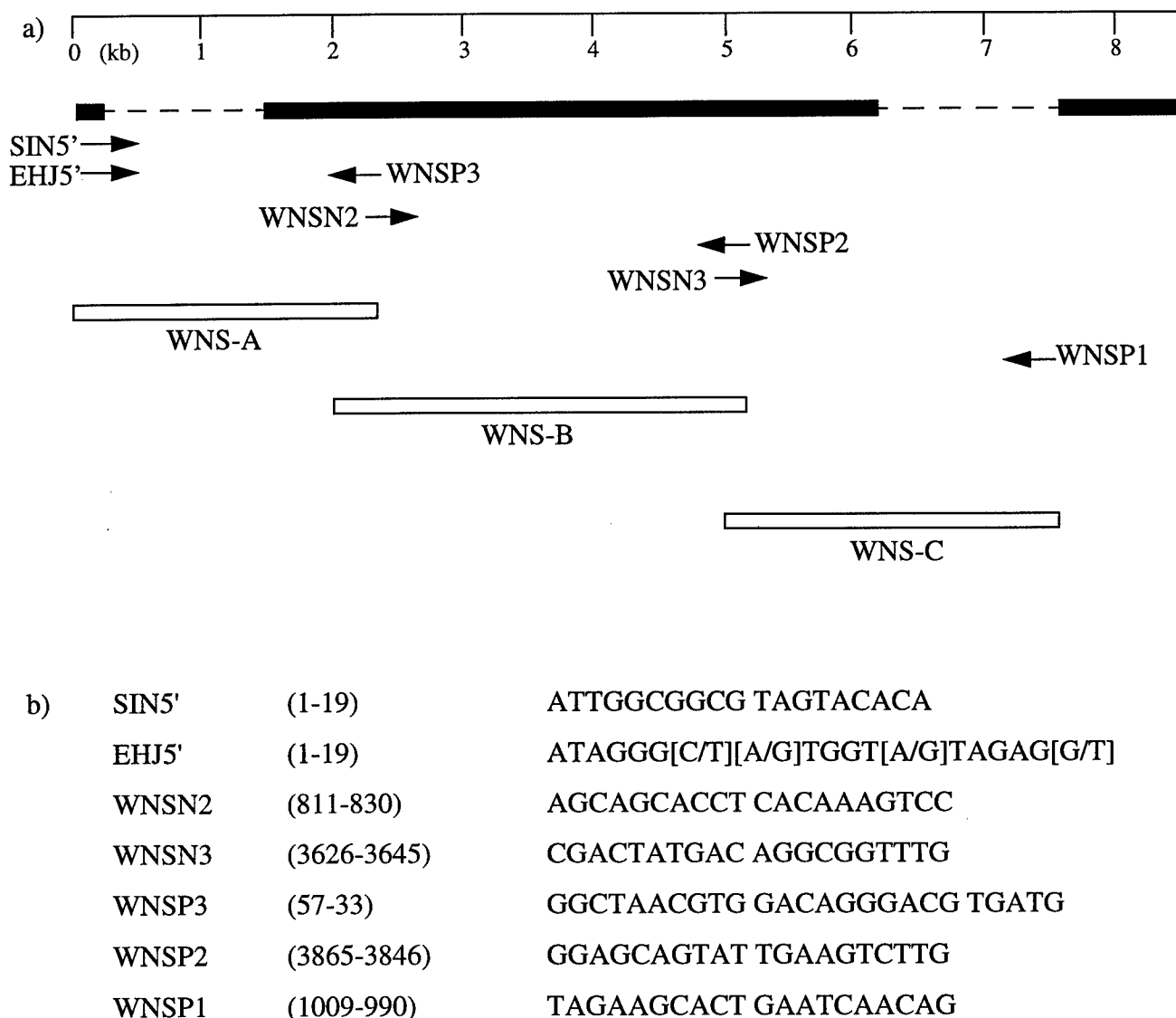


Figure 2. PCR Amplification of WEE Nonstructural Region

a) The RT-PCR cloning strategy is shown for WNS-A, B and C, together which encompass the entire nonstructural region of WEE. Primers used are indicated. b) The actual sequence of each of the primers is indicated, with the location in brackets: SIN5' and EHJ5' from Ou *et al*, 1983; WNSN2, WNSN3, WNSP1 and WNSP2 based on Weaver *et al*, 1994; and WNSP3 from Hahn, *et al*, 1988.

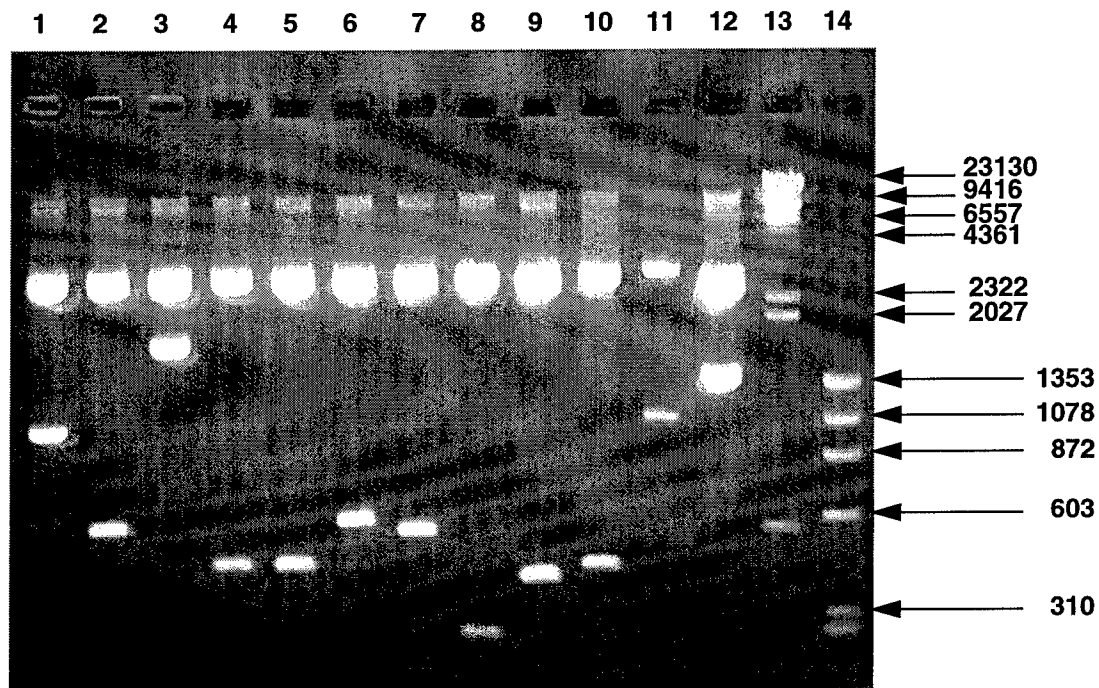


Figure 3. Restriction Analysis of HJ Isolates

Rapid plasmid preparations of HJ isolates were made up as described with the exception that a separate RNase A reaction was carried out before the final phenol/chloroform extraction and ethanol precipitation was completed. The washed and dried pellet of DNA was dissolved in 120 μ l of dH_2O . The restriction digest mixture consisted of 18 μ l DNA, 2 μ l 10x reaction buffer (500 mM Tris-HCl, pH8.0, 100 mM $MgCl_2$, 500 mM NaCl) and 2 μ l of *HindIII*/*XhoI* (2U of each) stock diluted in 1x reaction buffer. The tubes were incubated 3 hr at 37°C. The samples were run on a 1 % (w/v) TBE agarose gel. Lanes: 1-12) HJD-1 to HJD-12; 13) λ DNA/*HindIII* digested MW marker; 14) ϕ X174 RF DNA/*HaeIII* digested MW marker.

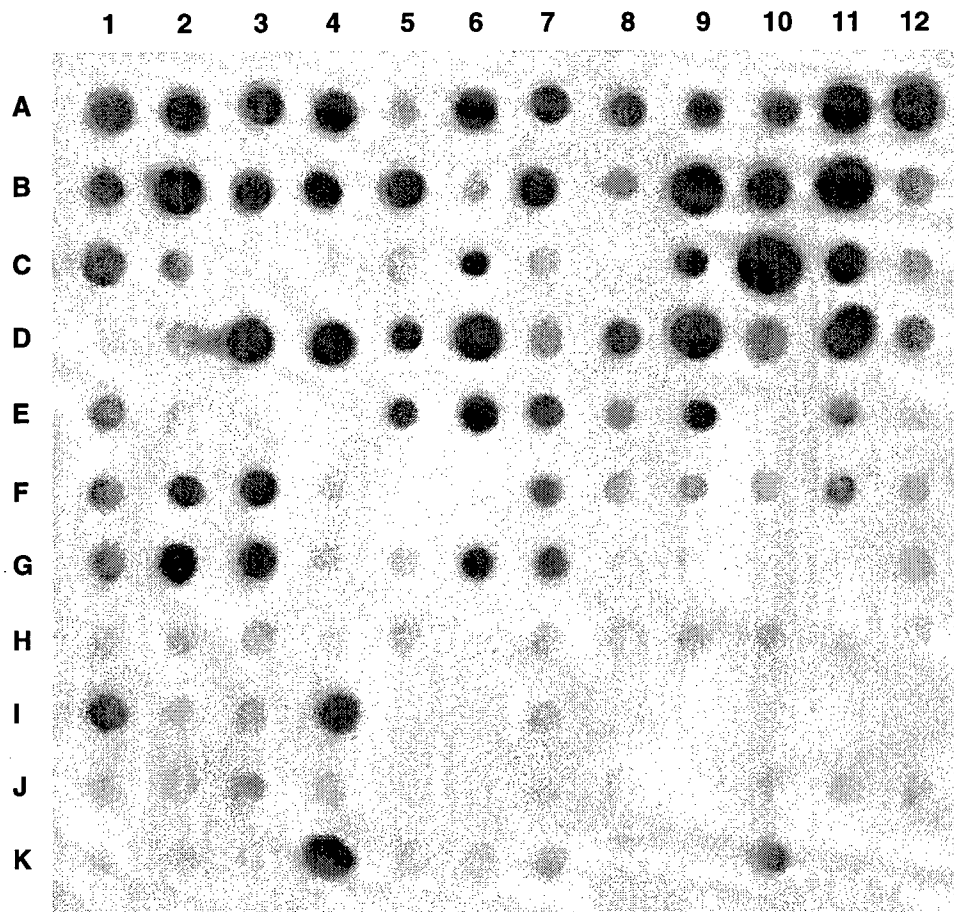


Figure 4. Dot Blot Hybridization of WEE Isolates

Rapid plasmid preparations of isolates from the WEE cDNA library (Invitrogen, San Diego, CA) were made up as described. Two μ l of each sample were spotted to a nylon membrane (Hybond-NTM - Amersham, Oakville, Ont.). Samples were air dried, UV fixed and hybridized with a Random primer [³²P]-labelled probe of the WEEN0/WEE3' PCR fragment as described. Samples: A1-12) pcDW 1-12; B1-12) pcDW 13-24; C1-12) pcDW 25-36; D1-12) pcDW37-48; E1-12) pcDW 49-60; F1-12) pcDW 61-72; G1-12) pcDW 73-84; H1-12) pcDW 85-96; I1-12) pcDW 97-108; J1-12) pcDW 109-120; K1-8) pcDW 121-128; K9) negative control (λ DNA/HindIII digested); K10) positive control (WEE 71V-1658 cDNA).

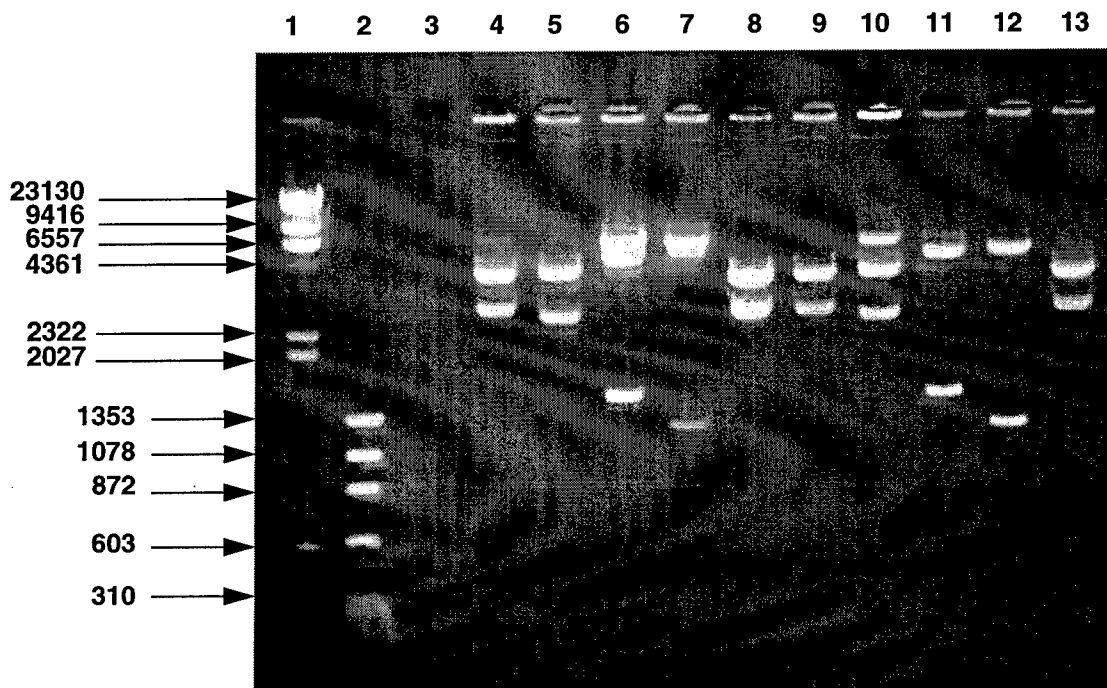


Figure 5. Restriction Mapping of WEE Isolates

Large-scale plasmid preparations were made to XH-7 and XH-12. Samples were digested with restriction enzymes as described and run on a 1 % (w/v) TBE agarose gel. Lanes: 1) λ DNA/HindIII digested MW marker; 2) Φ X174 RF DNA/HaeIII digested MW marker. XH-7 digested with: 4) *XbaI/HindIII*; 5) *XbaI/XhoI*; 6) *XbaI/NcoI*; 7) *BglII/NcoI*; 8) *SstI*. XH-12 digested with: 9) *XbaI/HindIII*; 10) *XbaI/XhoI*; 11) *XbaI/NcoI*; 12) *BglII/NcoI*; 13) *SstI*.

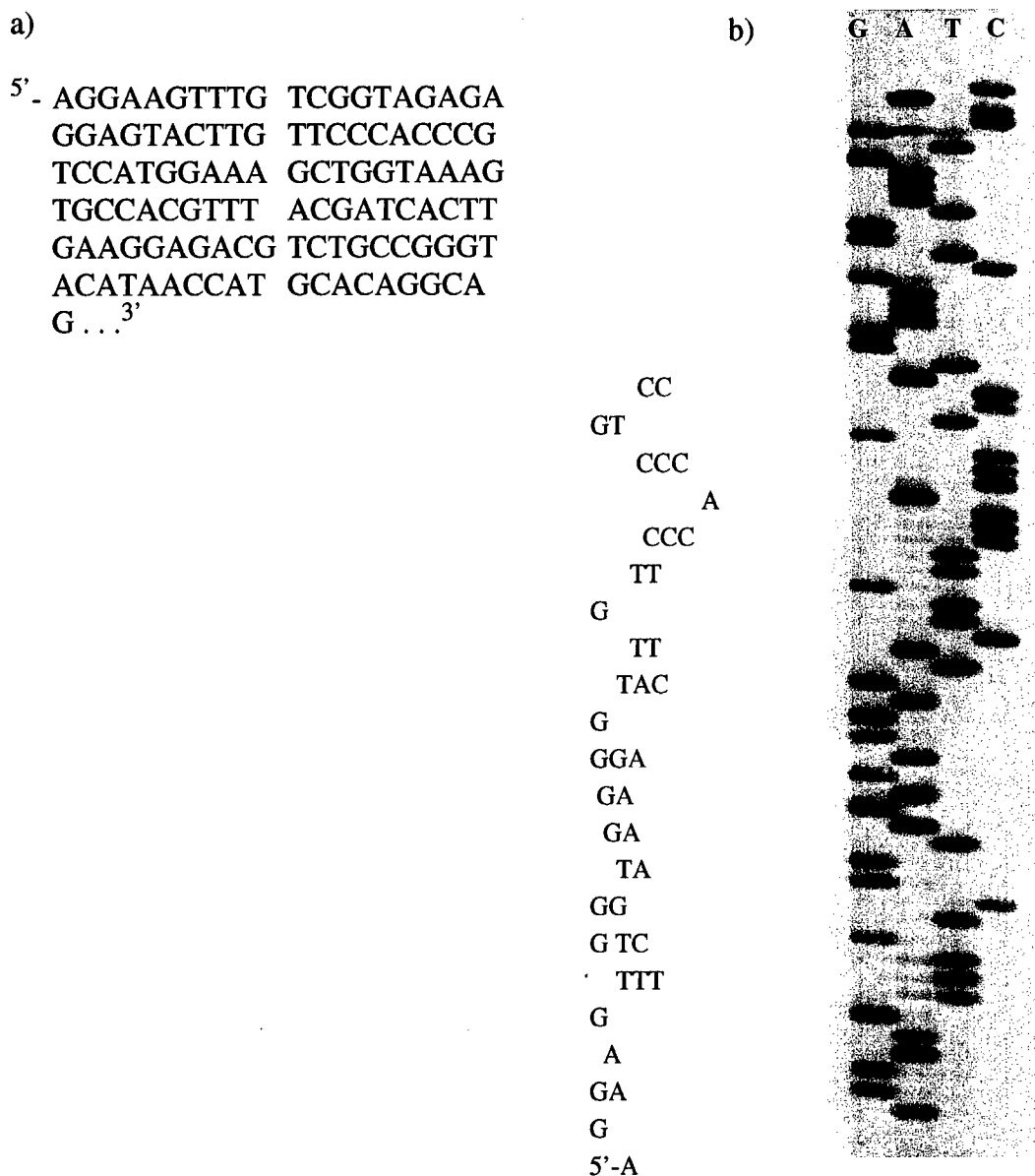


Figure 6. Manual DNA Sequence Analysis

Manual DNA sequencing was run as described, on a 8%(w/v) TBE gel (40 cm). The sequence is read sequentially from the bottom of the gel, using a set of four lanes. The sequence shown in a) used the WEEN4 primer to sequence WEE71V-1658 construct, XH-7; b) autoradiograph of the sequencing gel with the deduced sequence (5'-AGGAAGTTTGTCGGTAGAGAGAGGAG. . .)

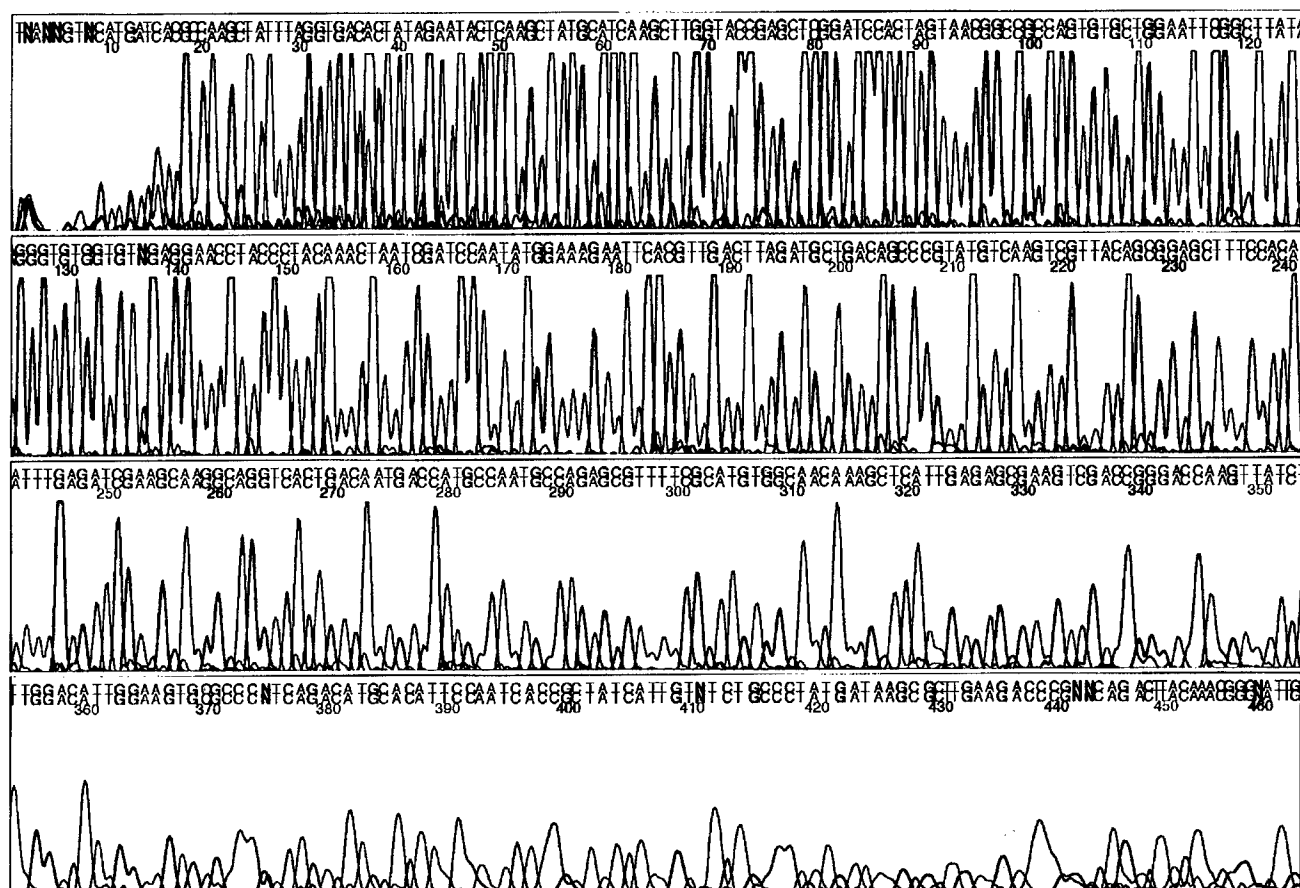


Figure 7. Fluorescent DNA Sequence Analysis

Automated sequencing was performed using a Applied Biosystem Instruments (ABI) 373 automated fluorescent sequencer, and the ABI Prism™ Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA) as described in Material and Methods. A chromatogram of DNA sequence data generated from WNS-TA3 using the M13 reverse primer is shown on the following page.

Position ¹	NT Change ²	Amino Acid Change ³	Protein
240	T g C	Cys g Arg (28)	NC
304	C g T	Ala g Val (49)	NC
424	A g G	Lys g Arg (89)	NC
907	A g G	Lys g Arg (250)	NC
1011	G g A	Ala g Thr (285)	E3
1182	G g A	Ala g Thr (342) (Ala in McMillan strain) ⁴	E2
1358	T g G	Asp g Glu (400)	E2
1984	C g T	Thr g Met (609)	E2
2055	A g T	Thr g Ser (633)	E2
2698	G g A	Arg g Lys (847) (Lys in McMillan strain) ⁴	E1
3669	A g T	Thr g Ser (1171)	E1

Residue 361 (a.a.) And 849 are Thr in the McMillan strain, but a Ser (361) and His (849), respectively, in 71V-1658 & BFS 1703⁴.

¹ Based on the 26S sequence of WEE strain BFS 1703 (Hahn *et al*, 1988).

² Nucleotide change (strain BFS 1703 g 71V-1658) at the position indicated.

³ Amino acid change (BFS 1703 g 71V-1658) at a.a. residue of 26S polyprotein ().

⁴ Comparison with protein sequence generated to the N-terminals of the NC, E2 & E1 proteins of the McMillan strain (Bell *et al*, 1983)

Figure 8. Structural Protein Homology Between WEE Strains

The deduced amino acid sequence of the structural proteins of WEE strain 71V-1658 was compared to the deduced amino acid sequence of BFS 1703 using the Clustal method of comparison (Lasergene sequence homology programs - DNA Star, Madison, WI). Silent mutations were not included.

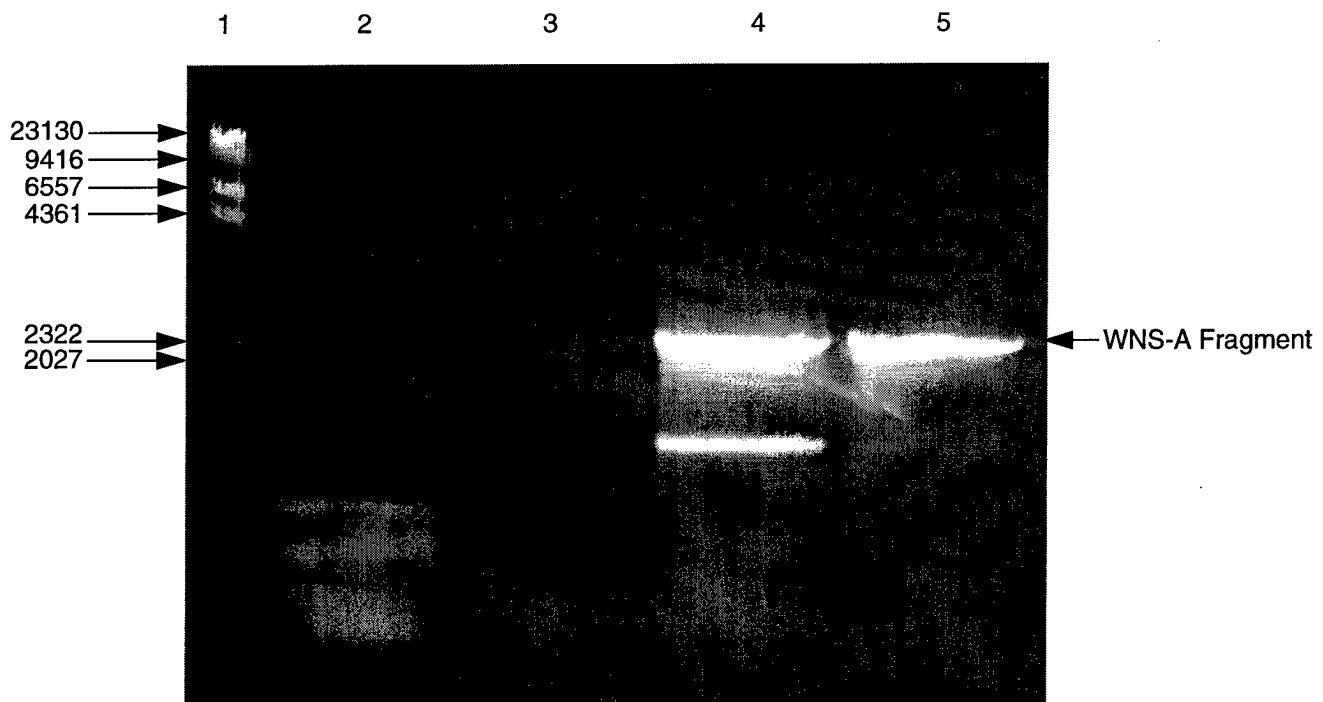


Figure 9. WEE 5' Nonstructural Region

RT-PCR reaction was run using WEE 71V-1658 RNA as template, and the following sets of primers with and without Perfect MatchTM (Stratagene, La Jolla, CA). a) RT-PCR reactions were run on a 1% preparatory low melting agarose gel. Lane: 1) λ *HindIII* digest; 2) SIN5'/WNSP3; 3) SIN5'/WNSP3 with Perfect MatchTM; 4) EHJ5'/WNSP3; 5) EHJ5'/WNSP3 with Perfect MatchTM.

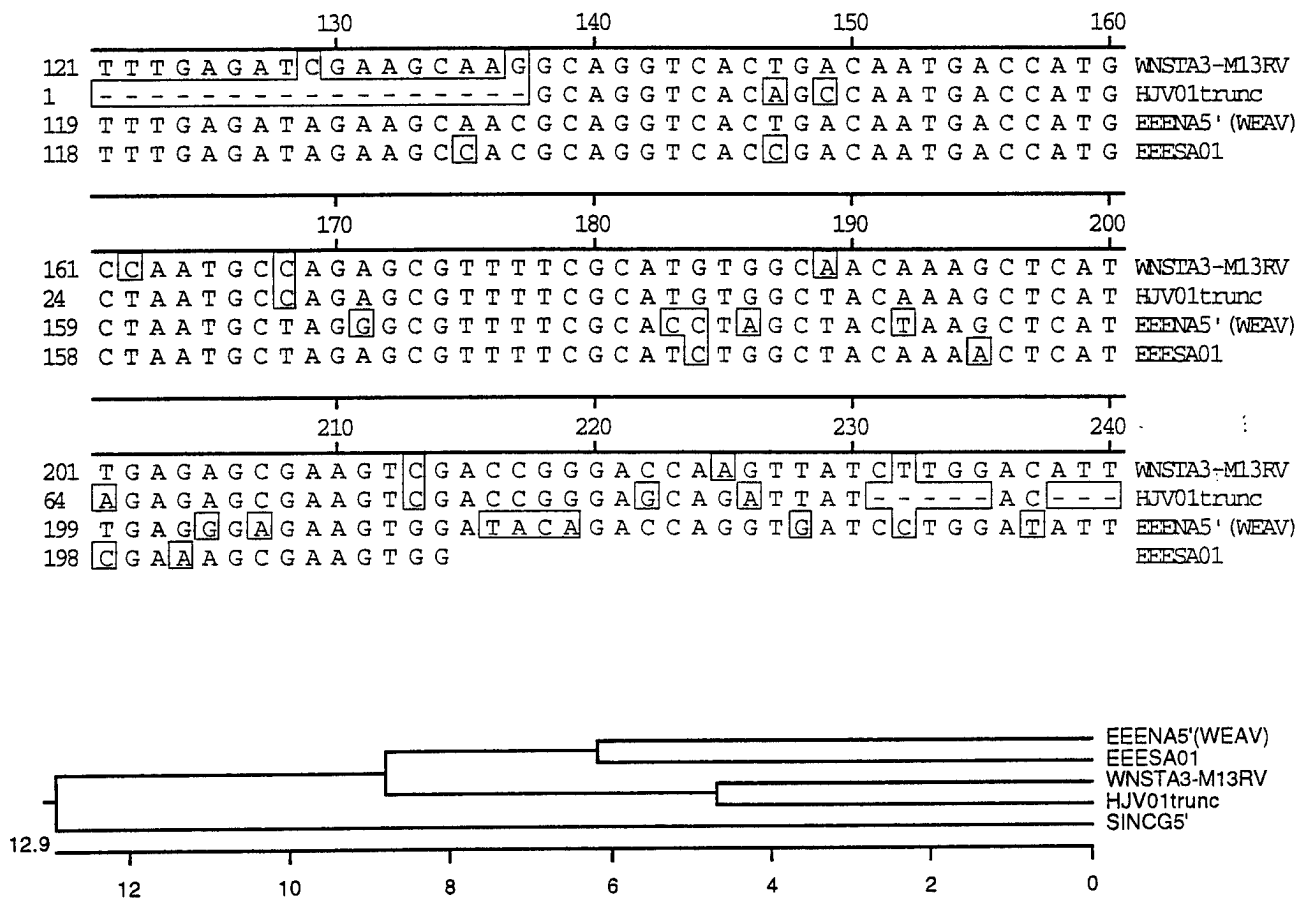


Figure 10. Nucleotide Homology In the WEE 5' Nonstructural Region

a) Sequence comparison of 5' end WEE 71V-1658 (WSNTA-3) with the HJ, EEE SA (Ou *et al*, 1983) and the EEE NA (Weaver *et al*, 1994) DNA sequences from nt 120-240, using the Clustal method; b) Phylogenetic tree of the DNA sequence comparison of 5' end of WEE, HJ, EEE SA, EEE NA and SIN HR (Strauss *et al*, 1984).

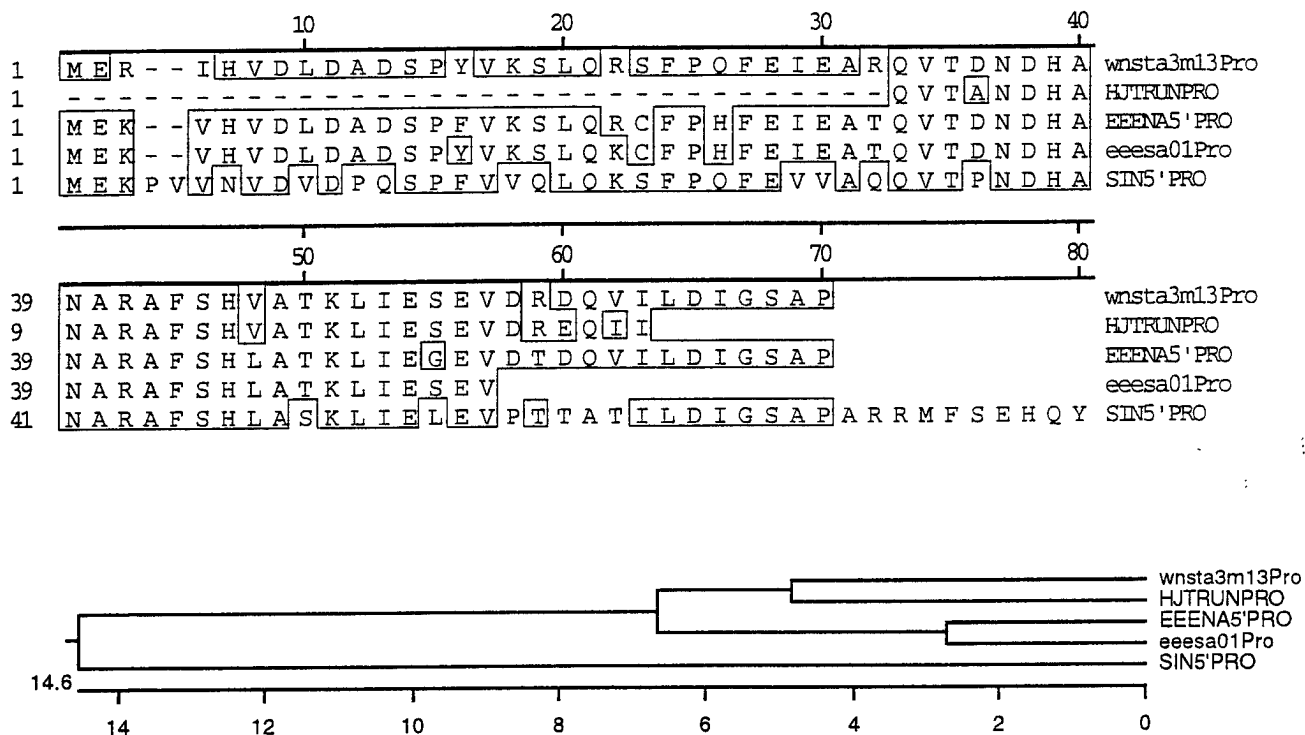


Figure 11. Homology of the N-terminus of Nonstructural Protein 1

a) Comparison of the translated protein sequence of WNSTA-3 sequence (N-terminal of nsp1) with HJ, EEE SA (Ou *et al*, 1983) and the EEE NA (Weaver *et al*, 1994) sequences using the Clustal method; b) Phylogenetic tree of the sequence comparison of the N-terminus of nsp1 of WEE, HJ, EEE SA, EEE NA and SIN HR (Strauss *et al*, 1984).

Appendix A Alignment of the 26S region of WEE strain 71V-1658 with BFS-1703 (Hahn, *et al*, 1988). The WEE sequence begins at nt 14 and ends at nt 4150. Some of the features are: the WEE 26S promoter (nt 101-124); the start of the 26S RNA (nt 120); the polyprotein start codon (nt 159); the termination codon (nt 3867); and the tandem repeats (nts 3896-3935, 3954-3993).

		10	20	30	40	
1	G G C C C T C T A	G A G C T C A T A C T	G G C A G G C C T	G A T C A T C A C G T	71V-1658seq1	
1	T C C A G A T A	C G A G A T C A T A C T	G G C A G G C C T	G A T C A T C A C G T	BFS-1703seq	
		50	60	70	80	
41	C T C T G T C C A C G T T	A G C C G A A A G C G T T	A A G A A C T T C A A G A G	71V-1658seq1		
41	C C C T G T C C A C G T T	A G C C G A A A G C G T T	A A G A A C T T C A A G A G	BFS-1703seq		
		90	100	110	120	
81	C A T A A G A G G G A	G C C C A A T C A C C C T	C T A C G G C T G A C C T	A A A	71V-1658seq1	
81	C A T A A G A G G G A	A C C C A A T C A C C C T	C T A C G G C T G A C C T	A A A	BFS-1703seq	
		130	140	150	160	
121	T A G G T G A C G T A G T	A G A C A C G C A C C T	A C C C A C C G C C A G A A T	71V-1658seq1		
121	T A G G T G A C G T A G T	A G A C A C G C A C C T	A C C C A C C G C C A A A A T	BFS-1703seq		
		170	180	190	200	
161	G T T T C C A T A C C C T	C A G C T G A A A C T T T	C C A C C A G T T T A C C C T	71V-1658seq1		
161	G T T T C C A T A C C C T	C A G C T G A A A C T T T	C C A C C A G T T T A C C C T	BFS-1703seq		
		210	220	230	240	
201	A C A A A T C C G A T G G C T T	A C C G A G A T C C A A A C C C T	C C T A G G C	C	71V-1658seq1	
201	A C A A A T C C G A T G G C T T	A C C G A G A T C C A A A C C C T	C C T A G G T		BFS-1703seq	
		250	260	270	280	
241	G C C G C T G G A G G C C G T T T	C G G C C C C G C T G G C T	G C T C A A A T	71V-1658seq1		
241	G C C G C T G G A G G C C G T T T	C G G C C C C G C T G G C T	G C T C A A A T	BFS-1703seq		
		290	300	310	320	
281	C G A A G A T C T T A G G A G G T C	G A T A G T C A A C T T	G A C T T T C A A A	71V-1658seq1		
281	C G A A G A T C T T A G G A G G T C	G A T A G C C A A C T T	A A C T T T C A A A	BFS-1703seq		
		330	340	350	360	
321	C A A C G A T C A C C T A A T C C G C C	G C C A G G T C C A C C G C C A A A G A	71V-1658seq1			
321	C A A C G A T C A C C T A A T C C G C C	G C C A G G T C C A C C G C C A A A G A	BFS-1703seq			
		370	380	390	400	
361	A G A A G A A G A G T G C T C C T A A G C C	A A A A C C T A C T C A G C C T A A	71V-1658seq1			
361	A G A A G A A G A G T G C T C C T A A G C C	A A A A C C T A C T C A G C C T A A	BFS-1703seq			
		410	420	430	440	
401	A A A G A A G A A G C A G C A A G C C A A G A G	G A C G A A A C G C A A G C C T	71V-1658seq1			
401	A A A G A A G A A G C A G C A A G C C A A G A A	G A C G A A A C G C A A G C C T	BFS-1703seq			

	450	460	470	480	
441	A A A C C A G G G A A A C G A C A A C G T A T G T G T A T G A A G T T G G A G T				71V-1658seq1
441	A A A C C A G G G A A A C G A C A G C G T A T G T G T A T G A A G T T G G A G T				BFS-1703seq
	490	500	510	520	
481	C G G A C A A G A C A T T T C C G A T C A T G C T G A A C G G C C A A G T G A A				71V-1658seq1
481	C G G A C A A G A C A T T T C C G A T C A T G C T G A A C G G C C A A G T G A A				BFS-1703seq
	530	540	550	560	
521	T G G A T A T G C C T G C G T T G T C G G A G G A A G G C T G A T G A A A C C A				71V-1658seq1
521	T G G A T A C G C T T G C G T T G T C G G A G G A A G G C T G A T G A A A C C A				BFS-1703seq
	570	580	590	600	
561	C T C C A C G T T G A A G G A A A A A T T G A T A A T G A G C A A T T A G C G G				71V-1658seq1
561	C T C C A C G T T G A A G G A A A A A T C G A T A A T G A G C A A T T A G C G G				BFS-1703seq
	610	620	630	640	
601	C C G T G A A A T T G A A G A A G G C T A G C A T G T A C G A C T T G G A G T A				71V-1658seq1
601	C C G T G A A A T T G A A G A A G G C T A G C A T G T A C G A C T T G G A G T A				BFS-1703seq
	650	660	670	680	
641	C G G C G A C G T T C C C C A G A A C A T G A A A T C A G A C A C G C T G C A G				71V-1658seq1
641	T G G C G A C G T T C C C C A G A A T A T G A A A T C A G A C A C G C T G C A G				BFS-1703seq
	690	700	710	720	
681	T A C A C C A G C G A C A A A C C A C C G G G C T T C T A C A A C T G G C A C C				71V-1658seq1
681	T A C A C C A G C G A C A A A C C A C C G G G C T T T T A C A A C T G G C A C C				BFS-1703seq
	730	740	750	760	
721	A C G G C G C A G T C C A G T A T G A G A A T G G G A G A T T T A C C G T A C C				71V-1658seq1
721	A C G G C G C A G T C C A G T A T G A G A A T G G G A G A T T C A C C G T A C C				BFS-1703seq
	770	780	790	800	
761	G A G A G G A G T G G G C G G G A A A G G C G A C A G C G G A A G A C C G A T C				71V-1658seq1
761	G A G A G G A G T G G G C G G G A A A G G C G A C A G T G G A A G A C C G A T C				BFS-1703seq
	810	820	830	840	
801	C T G G A C A A C A G A G G C A G A G T T G T G G C T A T T G T T C T A G G A G				71V-1658seq1
801	C T G G A C A A C A G A G G C A G A G T T G T G G C T A T T G T T C T A G G A G				BFS-1703seq
	850	860	870	880	
841	G T G C A A A T G A G G G C A C G C G T A C G G C G C T T T C A G T G G T C A C				71V-1658seq1
841	G T G C A A A C G A G G G C A C G C G T A C G G C G C T T T C A G T G G T C A C				BFS-1703seq

	890	900	910	920	
881	T T G G A A C C A G A A A G G G G T G A C C A T T A G G G A T A C C C C C G A A				71V-1658seq1
881	T T G G A A C C A G A A A G G G G T G A C C A T C A A G G A T A C C C C C G A A				BFS-1703seq
	930	940	950	960	
921	G G T T C T G A A C C G T G G T C A C T A G T T A C A G C G C T A T G C G T G C				71V-1658seq1
921	G G T T C T G A A C C G T G G T C A C T A G T T A C A G C G C T G T G C G T G C				BFS-1703seq
	970	980	990	1000	
961	T T T C G A A T G T C A C G T T C C C A T G C G A C A A A C C A C C C G T G T G				71V-1658seq1
961	T T T C G A A T G T C A C G T T C C C A T G C G A C A A A C C A C C C G T G T G				BFS-1703seq
	1010	1020	1030	1040	
1001	C T A T T C A C T G A C G C C A G A A C G A A C A C T C G A C G T G C T C G A A				71V-1658seq1
1001	C T A T T C A C T G G C G C C A G A A C G A A C A C T C G A C G T G C T C G A A				BFS-1703seq
	1050	1060	1070	1080	
1041	G A G A A C G T C G A C A A T C C A A A T T A C G A C A C G C T G C T G G A G A				71V-1658seq1
1041	G A G A A C G T C G A C A A T C C A A A T T A C G A C A C G C T G C T G G A G A				BFS-1703seq
	1090	1100	1110	1120	
1081	A C G T C T T G A A A T G T C C A T C A C G C C G G C C C A A A C G A A G C A T				71V-1658seq1
1081	A C G T C T T G A A A T G T C C A T C A C G C C G G C C C A A A C G A A G C A T				BFS-1703seq
	1130	1140	1150	1160	
1121	T A C C G A T G A C T T C A C A C T G A C C A G T C C C T A C C T G G G G T T C				71V-1658seq1
1121	T A C C G A T G A C T T C A C A C T G A C C A G T C C C T A C C T G G G G T T C				BFS-1703seq
	1170	1180	1190	1200	
1161	T G C C C G T A T T G C A G A C A C T C A A C G C C G T G T T T C A G C C C A A				71V-1658seq1
1161	T G C C C G T A T T G C A G A C A C T C A G C G C C G T G T T T C A G C C C A A				BFS-1703seq
	1210	1220	1230	1240	
1201	T A A A A A T T G A G A A C G T G T G G G A C G A A T C T G A T G A T G G A T C				71V-1658seq1
1201	T A A A A A T T G A G A A C G T G T G G G A C G A A T C T G A T G A T G G A T C				BFS-1703seq
	1250	1260	1270	1280	
1241	G A T T A G A A T C C A G G T C T C G G C A C A A T T C G G C T A C A A T C A G				71V-1658seq1
1241	G A T T A G A A T C C A G G T C T C G G C A C A A T T C G G C T A C A A T C A G				BFS-1703seq
	1290	1300	1310	1320	
1281	G C A G G C A C T G C G G A T G T C A C C A A A T T C C G T T A C A T G T C T T				71V-1658seq1
1281	G C A G G C A C T G C A G A T G T C A C C A A A T T C C G T T A C A T G T C T T				BFS-1703seq

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      1330      1340      1350      1360
1321  T C G A C C A C G A C C A T G A C A T C A A G G A A G A C A G T A T G G A G A A 71V-1658seq1
1321  T C G A C C A C G A C C A T G A C A T C A A G G A A G A C A G T A T G G A T A A BFS-1703seq

      1370      1380      1390      1400
1361  A A T A G C T A T C A G C A C A T C T G G A C C C T G C C G T C G T C T T G G C 71V-1658seq1
1361  A A T A G C T A T T A G C A C A T C T G G A C C C T G C C G T C G T C T T G G C BFS-1703seq

      1410      1420      1430      1440
1401  C A C A A A G G G T A C T T C C T G T T A G C T C A A T G T C C T C C A G G T G 71V-1658seq1
1401  C A C A A A G G G T A C T T C C T G T T A G C T C A A T G T C C T C C A G G T G BFS-1703seq

      1450      1460      1470      1480
1441  A C A G T G T A A C C G T C A G T A T C A C G A G C G G A G C A T C T G A G A A 71V-1658seq1
1441  A C A G T G T A A C C G T C A G T A T C A C G A G C G G A G C A T C T G A G A A BFS-1703seq

      1490      1500      1510      1520
1481  T T C A T G C A C C G T G G A G A A A A G A T C A G G A G G A A G T T T G T C 71V-1658seq1
1481  T T C A T G C A C C G T G G A G A A A A G A T C A G G A G G A A G T T T G T C BFS-1703seq

      1530      1540      1550      1560
1521  G G T A G A G A G G A G T A C T T G T T C C C A C C C G T C C A T G G A A A G C 71V-1658seq1
1521  G G T A G A G A G G A G T A C T T G T T C C C A C C C G T C C A T G G A A A G C BFS-1703seq

      1570      1580      1590      1600
1561  T G G T A A A G T G C C A C G T T T A C G A T C A C T T G A A G G A G A C G T C 71V-1658seq1
1561  T G G T A A A G T G C C A C G T T T A C G A T C A C T T G A A G G A G A C G T C BFS-1703seq

      1610      1620      1630      1640
1601  T G C C G G G T A C A T A A C C A T G C A C A G G C C A G G C C C A C A C G C G 71V-1658seq1
1601  T G C C G G A T A C A T A A C C A T G C A C A G G C C A G G C C C A C A C G C G BFS-1703seq

      1650      1660      1670      1680
1641  T A T A A G T C C T A T C T G G A G G A A G C G T C A G G C G A A G T G T A C A 71V-1658seq1
1641  T A T A A G T C C T A T C T G G A G G A A G C G T C A G G C G A A G T G T A C A BFS-1703seq

      1690      1700      1710      1720
1681  T T A A A C C A C C T T C T G G C A A G A A C G T C A C C T A C G A A T G T A A 71V-1658seq1
1681  T T A A A C C A C C T T C T G G C A A G A A C G T C A C C T A C G A A T G T A A BFS-1703seq

      1730      1740      1750      1760
1721  G T G T G G C G A C T A C A G C A C A G G T A T C G T G A G C A C G C G A A C G 71V-1658seq1
1721  G T G T G G C G A C T A C A G C A C A G G T A T T G T G A G C A C G C G A A C G BFS-1703seq

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	1770	1780	1790	1800	
1761	A A G A T G A A C G G C T G C A C T A A A G C A A A A C A G T G C A T T G C C T				71V-1658seq1
1761	A A G A T G A A C G G C T G C A C T A A A G C A A A A C A G T G C A T T G C C T				BFS-1703seq
	1810	1820	1830	1840	
1801	A C A A G A G C G A C C A A A C G A A A T G G G T C T T C A A C T C G C C G G A				71V-1658seq1
1801	A C A A G A G C G A C C A A A C G A A A T G G G T C T T C A A C T C G C C G G A				BFS-1703seq
	1850	1860	1870	1880	
1841	T C T T A T T A G G C A C A C A G A C C A C T C A G T G C A A G G T A A A T T G				71V-1658seq1
1841	T C T T A T T A G G C A C A C A G A C C A C T C A G T G C A A G G T A A A C T G				BFS-1703seq
	1890	1900	1910	1920	
1881	C A C A T T C C A T T T C G C T T G A C A C C G A C A G T C T G C C C G G T T C				71V-1658seq1
1881	C A C A T T C C A T T T C G C T T G A C A C C G A C A G T C T G C C C G G T T C				BFS-1703seq
	1930	1940	1950	1960	
1921	C G T T A G C T C A C A C G C C T A C A G T C A C G A A G T G G T T C A A A G G				71V-1658seq1
1921	C G T T A G C T C A C A C G C C T A C A G T C A C G A A G T G G T T C A A A G G				BFS-1703seq
	1970	1980	1990	2000	
1961	C A T C A C C C T C C A C C T G A C T G C A A T G C G A C C A A C A T T G C T G				71V-1658seq1
1961	C A T C A C C C T C C A C C T G A C T G C A A C G C G A C C A A C A T T G C T G				BFS-1703seq
	2010	2020	2030	2040	
2001	A C A A C G A G A A A A T T G G G G C T G C G A G C A G A C G C A A C A G C A G				71V-1658seq1
2001	A C A A C G A G A A A A T T G G G G C T G C G A G C A G A C G C A A C A G C A G				BFS-1703seq
	2050	2060	2070	2080	
2041	A A T G G A T T A C A G G G T C T A C A T C C A G G A A T T T T T C T G T G G G				71V-1658seq1
2041	A A T G G A T T A C A G G G A C T A C A T C C A G G A A T T T T T C T G T G G G				BFS-1703seq
	2090	2100	2110	2120	
2081	G C G A G A A G G G C T G G A G T A C G T A T G G G G T A A C C A T G A A C C A				71V-1658seq1
2081	G C G A G A A G G G C T G G A G T A C G T A T G G G G C A A C C A T G A A C C A				BFS-1703seq
	2130	2140	2150	2160	
2121	G T C A G A G T C T G G G C C C A G G A G T C G G C A C C A G G C G A C C C A C				71V-1658seq1
2121	G T C A G A G T C T G G G C C C A G G A G T C G G C A C C A G G C G A C C C A C				BFS-1703seq
	2170	2180	2190	2200	
2161	A T G G A T G G C C G C A T G A G A T C A T C A T C C A C T A T T A T C A T C G				71V-1658seq1
2161	A T G G A T G G C C G C A T G A G A T C A T C A T C C A C T A T T A T C A T C G				BFS-1703seq

	2210	2220	2230	2240	
2201	G C A T C C A G T C T A C A C T G T C A T T G T G C T G T G T G G T G T C G C T				71V-1658seq1
2201	G C A T C C A G T C T A C A C T G T C A T T G T G C T G T G T G G T G T C G C T				BFS-1703seq
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	2250	2260	2270	2280	
2241	C T T G C T A T C C T G G T A G G C A C T G C A T C A T C A G C A G C T T G C A				71V-1658seq1
2241	C T T G C T A T C C T G G T A G G C A C T G C A T C G T C A G C A G C T T G T A				BFS-1703seq
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	2290	2300	2310	2320	
2281	T C G C C A A A G C A A G A A G A G A C T G C C T G A C G C C A T A C G C G C T				71V-1658seq1
2281	T C G C C A A A G C A A G A A G A G A C T G C C T G A C G C C A T A C G C G C T				BFS-1703seq
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	2330	2340	2350	2360	
2321	T G C A C C G A A C G C A A C G G T A C C C A C A G C A T T A G C G G T T T T G				71V-1658seq1
2321	T G C A C C G A A C G C A A C G G T A C C C A C A G C A T T A G C A G T T T T G				BFS-1703seq
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	2370	2380	2390	2400	
2361	T G C T G C A T T C G G C C A A C C A A C G C T G A A A C A T T T G G A G A A A				71V-1658seq1
2361	T G C T G T A T T C G G C C A A C C A A C G C T G A A A C A T T T G G A G A A A				BFS-1703seq
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	2410	2420	2430	2440	
2401	C T T T G A A C C A T C T G T G G T T T A A C A A C C A A C C G T T T C T C T G				71V-1658seq1
2401	C T T T G A A C C A T C T G T G G T T T A A C A A C C A A C C G T T T C T C T G				BFS-1703seq
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	2450	2460	2470	2480	
2441	G G C A C A G T T G T G C A T T C C T C T G G C A G C G C T T G T T A T T C T G				71V-1658seq1
2441	G G C A C A G T T G T G C A T C C C T C T G G C A G C G C T T G T T A T T C T G				BFS-1703seq
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	2490	2500	2510	2520	
2481	T T C C G C T G C T T T T C A T G C T G C A T G C C T T T T T A T T G G T T G				71V-1658seq1
2481	T T C C G C T G C T T T T C A T G C T G C A T G C C T T T T T A T T G G T T G				BFS-1703seq
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	2530	2540	2550	2560	
2521	C A G G C G T C T G C C T G G G G A A G G T A G A C G C C T T C G A A C A T G C				71V-1658seq1
2521	C A G G C G T C T G C C T G G G G A A G G T A G A C G C C T T C G A A C A T G C				BFS-1703seq
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	2570	2580	2590	2600	
2561	G A C C A C T G T G C C A A A T G T T C C G G G G A T C C C G T A T A A G G C G				71V-1658seq1
2561	G A C C A C T G T G C C A A A T G T T C C G G G G A T C C C G T A T A A G G C G				BFS-1703seq
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	2610	2620	2630	2640	
2601	T T G G T C G A A C G C G C A G G T T A C G C G C C A C T T A A C T G G A G A				71V-1658seq1
2601	T T G G T C G A A C G T G C A G G T T A C G C G C C A C T T A A T C T G G A G A				BFS-1703seq

	2650	2660	2670	2680		
2641	T C A C G	G T C G T C T C A T C G G A A T T A A C A C C T	T C A A C T A A C A A	71V-1658seq1		
2641	T C A C T G T C G T C T C A T C G G A A T T A A C A C C	C	T C A A C T A A C A A	BFS-1703seq		
	2690	2700	2710	2720		
2681	G G A G T A C G T G A C C T G C A	A	A T T C C A C A C A G T C A T T C C T T C A	71V-1658seq1		
2681	G G A G T A C G T G A C C T G C A G A T T C C A C A C A G T C A T T C C T T C A			BFS-1703seq		
	2730	2740	2750	2760		
2721	C C A C A A G T T A A A T G C T G C G G G T C C C T C G A G T G	C	A A G G C A T	71V-1658seq1		
2721	C C A C A A G T T A A A T G C T G C G G G T C C C T C G A G T G T A A G G C A T			BFS-1703seq		
	2770	2780	2790	2800		
2761	C C T C A A A G G C G G A T T A C A C A T G C C G C G T T T T T G G C G G T G T			71V-1658seq1		
2761	C C T C A A A	A	G C G G A T T A C A C A T G C C G C G T T T T T G G C G G T G T	BFS-1703seq		
	2810	2820	2830	2840		
2801	G T A C C C T T T C A T G T G G G G A G G C G C A C A	A	T G C T T C T G T G A C	71V-1658seq1		
2801	G T A C C C T T T C A T G T G G G G A G G C G C A C A G T G C T T C T G T G A C			BFS-1703seq		
	2850	2860	2870	2880		
2841	A G T G A G A A C A C A C A A C T G A G T G A G G C G T A C G T C G A G T T C G			71V-1658seq1		
2841	A G T G A G A A C A C A C A A C T G A G T G A G G C	A	T A C G T C G A G T T C G	BFS-1703seq		
	2890	2900	2910	2920		
2881	C T C C A G A C T G C A C T A T	A	G A T C A	C	G C A G T C G C A C T A A A A G T	71V-1658seq1
2881	C T C C A G A C T G C A C T A T T G A T C A T G C A G T C G C A C T A A A A G T					BFS-1703seq
	2930	2940	2950	2960		
2921	T C A C A C A G C T G C T C T G A A A G T C G G C C T G C G T A T A G T A T A C			71V-1658seq1		
2921	T C A C A C A G C T G C T C T G A A A G T C G G C C T G C G T A T A G T A T A C			BFS-1703seq		
	2970	2980	2990	3000		
2961	G G C A A	C	A C C A C C G C G C A C C T G G A T A C G T T T	T	G T C A A T G G C G	71V-1658seq1
2961	G G C A A T A C C A C C G C G C A C C T G G A T A C G T T	C	G T C A A T G G C G			BFS-1703seq
	3010	3020	3030	3040		
3001	T C A C G C C A G G T T C C T C A C G G G A C C T G A A G G T C A T A G C A G G			71V-1658seq1		
3001	T C A C G C C A G G T T C C T C A C G G G A C C T G A A G G T C A T A G C A G G			BFS-1703seq		
	3050	3060	3070	3080		
3041	G C C G A T A T C A G C C G C T T T T T C A C C C T T T G A C C A T A A G G T C			71V-1658seq1		
3041	G C C G A T A T C A G C C G C T T T T T C A C C C T T T G A C C A T A A G G T C			BFS-1703seq		

	3090	3100	3110	3120	
3081	G T C A T C A G A A A G G G G C T T G T T T A C A A C T A C G A C T T C C C T G				71V-1658seq1
3081	G T C A T C A G A A A G G G G C T T G T T T A C A A C T A C G A C T T C C C T G				BFS-1703seq
	3130	3140	3150	3160	
3121	A G T A T G G A G C T A T G A A A C C A G G A G C G T T C G G C G A T A T T C A				71V-1658seq1
3121	A <u>A</u> T A T G G A G C T A T G A A A C C A G G A G C G T T C G G C G A T A T T C A				BFS-1703seq
	3170	3180	3190	3200	
3161	A G C A T C C T C G C T T G A T G C T A C A G A C A T A G T A G C C C G C A C T				71V-1658seq1
3161	A G C A T C C T C G C T T G A T G C <u>C</u> A C A G A C A T A G T A G C C C G C A C T				BFS-1703seq
	3210	3220	3230	3240	
3201	G A C A T A C G G C T G C T G A A G C C T T C T G T C A A G A A C A T C C A C G				71V-1658seq1
3201	G A C A T A C G G C T G C T G A A G C C T T C T G T C A A G A A C A T C C A C G				BFS-1703seq
	3250	3260	3270	3280	
3241	T C C C C T A C A C C C A A G C A G T A T C A G G G T A T G A A A T G T G G A A				71V-1658seq1
3241	T C C C C T A C A C C C A A G C A G T A T C A G G G T A T G A A A T G T G G A A				BFS-1703seq
	3290	3300	3310	3320	
3281	G A A C A A C T C A G G A C G A C C C C T G C A A G A A A C A G C A C C A T T T				71V-1658seq1
3281	G A A C A A C T C A G G A C G A C C C C T G C A A G A A A C A G C A C C A T T T				BFS-1703seq
	3330	3340	3350	3360	
3321	G G A T G T A A A A T T G A A G T G G A G C C T C T G C G A G C G T C T A A C T				71V-1658seq1
3321	G G A T G T A A A A T T G A A G T G G A G C C T C T G C G A G C G T C T A A C T				BFS-1703seq
	3370	3380	3390	3400	
3361	G T G C T T A C G G G C A C A T C C C T A T C T C G A T T G A C A T C C C T G A				71V-1658seq1
3361	G T G C T T A C G G G C A C A T C C C T A T C T C G A T T G A C A T C C C T G A				BFS-1703seq
	3410	3420	3430	3440	
3401	T G C A G C T T T T G T G A G A T C A T C A G A A T C A C C A A C A A T T T T A				71V-1658seq1
3401	<u>C</u> G C A G C T T T T G T G A G A T C A T C A G A A T C A C C A A C A A T T T T A				BFS-1703seq
	3450	3460	3470	3480	
3441	G A A G T T A G C T G C A C A G T A G C A G A C T G C A T T T A T T C T G C A G				71V-1658seq1
3441	G A A G T T A G C T G C A C A G T A G C A G A C T G C A T T T A T T C T G C A G				BFS-1703seq
	3490	3500	3510	3520	
3481	A C T T T G G T G G T T C T C T A A C A T T A C A G T A C A A A G C T G A C A G				71V-1658seq1
3481	A C T T T G G T G G T T C <u>G</u> C T A A C A T T A C A G T A C A A A G C T G A C A G				BFS-1703seq

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      3530      3540      3550      3560
3521 G G A G G G A C A T T G T C C A G T T C A C T C C C A C T C C A C G A C A G C T 71V-1658seq1
3521 G G A G G G A C A T T G T C C A G T T C A C T C C C A C T C C A C A A C A C A G C T BFS-1703seq

      3570      3580      3590      3600
3561 G T T T T G A A G G A A G C G A C C A C A C A T G T G A C T G C C G T A G G C A 71V-1658seq1
3561 G T T T T G A A G G A A G C G A C C A C A C A T G T G A C T G C C G T A G G C A BFS-1703seq

      3610      3620      3630      3640
3601 G C A T A A C A C T A C A T T T T A G C A C A T C G A G C C C A C A A G C A A A 71V-1658seq1
3601 G C A T A A C A C T A C A T T T T A G C A C A T C G A G C C C A C A A G C A A A BFS-1703seq

      3650      3660      3670      3680
3641 T T T T A T A G T T T C G C T A T G C G G C A A G A A G T C C A C C T G C A A T 71V-1658seq1
3641 T T T T A T A G T T T C G C T A T G C G G C A A G A A G A C C A C C T G C A A T BFS-1703seq

      3690      3700      3710      3720
3681 G C T G A A T G T A A A C C A C C G G C C G A C C A C A T A A T T G G A G A A C 71V-1658seq1
3681 G C T G A A T G T A A A C C A C C G G C C G A C C A C A T A A T T G G A G A A C BFS-1703seq

      3730      3740      3750      3760
3721 C A C A T A A A G T C G A C C A A G A A T T C C A G G C G G C A G T T T C C A A 71V-1658seq1
3721 C A C A T A A A G T C G A C C A A G A A T T C C A G G C G G C A G T T T C C A A BFS-1703seq

      3770      3780      3790      3800
3761 A A C A T C T T G G A A T T G G C T G C T T G C A C T G T T T G G G G G A G C A 71V-1658seq1
3761 A A C A T C T T G G A A T T G G C T G C T T G C A C T G T T T G G G G G A G C A BFS-1703seq

      3810      3820      3830      3840
3801 T C A T C C C T C A T T G T T G T A G G A C T T A T A G T G T T G G T C T G C A 71V-1658seq1
3801 T C A T C C C T C A T T G T T G T A G G A C T T A T A G T G T T G G T C T G C A BFS-1703seq

      3850      3860      3870      3880
3841 G C T C T A T G C T T A T A A A C A C A C G T A G A T G A C T G A G C G C G G A 71V-1658seq1
3841 G C T C T A T G C T T A T A A A C A C A C G T A G A T G A C T G A G C G C G G A BFS-1703seq

      3890      3900      3910      3920
3881 C A C T G A C A T A G C G G T A A A A C T C G A T G T A C T T C C G A G G A A G 71V-1658seq1
3881 C A C T G A C A T A G C G G T A A A A C T C G A T G T A C T T C C G A G G A A G BFS-1703seq

      3930      3940      3950      3960
3921 C G T G G T G C A T A A T G C C A C G C G C C G C T T G A C A C T A A A A C T C 71V-1658seq1
3921 C G T G G T G C A T A A G C C A C G C G C C G C T T G A C A C T A A A A C T C BFS-1703seq
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	3970	3980	3990	4000	
3961	G A T G T A T T T C C G A G G A A G C A C A G T G C A T A A T G C T G T G C A G				71V-1658seq1
3961	G A T G T A T T T C C G A G G A A G C A C A G T G C A T A A T G C T G T G C A G				BFS-1703seq
<hr/>					
	4010	4020	4030	4040	
4001	T G T C A C A T T A A T C G T A T A T C A C A C T A C A T A T T A A C A A C A C				71V-1658seq1
4001	T G T C A C A T T A A T C G T A T A T C A C A C T A C A T A T T A A C A A C A C				BFS-1703seq
<hr/>					
	4050	4060	4070	4080	
4041	T A T A T C A C T T T T A T G A G A C T C A C T A T G G G T C T C T A A T A T A				71V-1658seq1
4041	T A T A T C A C T T T T A T G A G A C T C A C T A T G G G T C T C T A A T A T A				BFS-1703seq
<hr/>					
	4090	4100	4110	4120	
4081	C A C T A C A C A T A T T T A T A C T T A A A A A C A C T A T A C A C A C T T C A				71V-1658seq1
4081	C A C T A C A C A T A T T T T A C T T A A A A A C A C T A T A C A C A C T T T A				BFS-1703seq
<hr/>					
	4130	4140	4150	4160	
4121	T A A A T T C T C T C A T A A T T T C A C T T T - - - - -				71V-1658seq1
4121	T A A A T T C T T T T A T A A T T T T C T T T T G T T T T A T T T T G T T T				BFS-1703seq
<hr/>					
	4170				
4145	- - - A G G T T T				71V-1658seq1
4161	T T A A A A T T T C				BFS-1703seq

Decoration 'Decoration #1': Box residues that differ from the Consensus.

Appendix B Alignment of the amino acid sequences of the 26S region of WEE strain 71V-1658 and BFS-1703.

	10	20	30	40		
1	MFPYPQLNFP	PVYPTNPM	AYRDPNPP	RRRWRPFR	PPLAAQ 71V-1658prt	
1	MFPYPQLNFP	PVYPTNPM	AYRDPNPP	RCRWRPFR	PPLAAQ BFS-1703prt	
	50	60	70	80		
41	IEDLRRSI	IVNLTFKQRS	PNPPPGPPP	KKKKKSAP	KPKPTQP 71V-1658prt	
41	IEDLRRSI	IANLTFKQRS	PNPPPGPPP	KKKKKSAP	KPKPTQP BFS-1703prt	
	90	100	110	120		
81	KKKKQQAK	RTKRKPKPG	KRQRMCMK	LES DKT	FPIMLNGQV 71V-1658prt	
81	KKKKQQAK	RTKRKPKPG	KRQRMCMK	LES DKT	FPIMLNGQV BFS-1703prt	
	130	140	150	160		
121	NGYACVVGG	RRLMKPLH	VEGKIDNE	QLAAVKL	KKASMYDLE 71V-1658prt	
121	NGYACVVGG	RRLMKPLH	VEGKIDNE	QLAAVKL	KKASMYDLE BFS-1703prt	
	170	180	190	200		
161	YGDVPQNM	KSDTLQYT	SDKPPGF	YNWHHG	AVQYENGRFTV 71V-1658prt	
161	YGDVPQNM	KSDTLQYT	SDKPPGF	YNWHHG	AVQYENGRFTV BFS-1703prt	
	210	220	230	240		
201	PRGVGGK	GDSGRPIL	DNRGRV	VAIVLGG	ANEGTRTALS VV 71V-1658prt	
201	PRGVGGK	GDSGRPIL	DNRGRV	VAIVLGG	ANEGTRTALS VV BFS-1703prt	
	250	260	270	280		
241	TWNQKGV	TIIRDTPE	GSEPW	SLVTAL	CVLSNVTFPCDKPPV 71V-1658prt	
241	TWNQKGV	TIKDTPE	GSEPW	SLVTAL	CVLSNVTFPCDKPPV BFS-1703prt	
	290	300	310	320		
281	CYSLIT	PERTLDV	LEENV	DNPNYD	TLL ENVLKCP	SRRPKRS 71V-1658prt
281	CYSLA	PERTLDV	LEENV	DNPNYD	TLL ENVLKCP	SRRPKRS BFS-1703prt
	330	340	350	360		
321	ITDDFTLT	SPYLGFC	PYCRHS	ITPCF	SPIKIEN	VWDESDDG 71V-1658prt
321	ITDDFTLT	SPYLGFC	PYCRHS	A	PCFSP	IKIENVWDESDDG BFS-1703prt
	370	380	390	400		
361	SIRIQV	SAQFGYN	QAGTADV	TKFRY	MSFDH	DHDIKEDSME 71V-1658prt
361	SIRIQV	SAQFGYN	QAGTADV	TKFRY	MSFDH	DHDIKEDSMD BFS-1703prt
	410	420	430	440		
401	KIAIST	SGPCRRL	GHKG	YFLLA	QC	PPGDSVTVSITSGASE 71V-1658prt
401	KIAIST	SGPCRRL	GHKG	YFLLA	QC	PPGDSVTVSITSGASE BFS-1703prt

	450	460	470	480	
441	N S C T V E K K I R R K F V G R E E Y L F P P V H G K L V K C H V Y D H L K E T				71V-1658prt
441	N S C T V E K K I R R K F V G R E E Y L F P P V H G K L V K C H V Y D H L K E T				BFS-1703prt
	490	500	510	520	
481	S A G Y I T M H R P G P H A Y K S Y L E E A S G E V Y I K P P S G K N V T Y E C				71V-1658prt
481	S A G Y I T M H R P G P H A Y K S Y L E E A S G E V Y I K P P S G K N V T Y E C				BFS-1703prt
	530	540	550	560	
521	K C G D Y S T G I V S T R T K M N G C T K A K Q C I A Y K S D Q T K W V F N S P				71V-1658prt
521	K C G D Y S T G I V S T R T K M N G C T K A K Q C I A Y K S D Q T K W V F N S P				BFS-1703prt
	570	580	590	600	
561	D L I R H T D H S V Q G K L H I P F R L T P T V C P V P L A H T P T V T K W F K				71V-1658prt
561	D L I R H T D H S V Q G K L H I P F R L T P T V C P V P L A H T P T V T K W F K				BFS-1703prt
	610	620	630	640	
601	G I T L H L T A <u>M</u> R P T L L T T R K L G L R A D A T A E W I T G <u>S</u> T S R N F S V				71V-1658prt
601	G I T L H L T A T R P T L L T T R K L G L R A D A T A E W I T G <u>T</u> T S R N F S V				BFS-1703prt
	650	660	670	680	
641	G R E G L E Y V W G N H E P V R V W A Q E S A P G D P H G W P H E I I I H Y Y H				71V-1658prt
641	G R E G L E Y V W G N H E P V R V W A Q E S A P G D P H G W P H E I I I H Y Y H				BFS-1703prt
	690	700	710	720	
681	R H P V Y T V I V L C G V A L A I L V G T A S S A A C I A K A R R D C L T P Y A				71V-1658prt
681	R H P V Y T V I V L C G V A L A I L V G T A S S A A C I A K A R R D C L T P Y A				BFS-1703prt
	730	740	750	760	
721	L A P N A T V P T A L A V L C C I R P T N A E T F G E T L N H L W F N N Q P F L				71V-1658prt
721	L A P N A T V P T A L A V L C C I R P T N A E T F G E T L N H L W F N N Q P F L				BFS-1703prt
	770	780	790	800	
761	W A Q L C I P L A A L V I L F R C F S C C M P F L L V A G V C L G K V D A F E H				71V-1658prt
761	W A Q L C I P L A A L V I L F R C F S C C M P F L L V A G V C L G K V D A F E H				BFS-1703prt
	810	820	830	840	
801	A T T V P N V P G I P Y K A L V E R A G Y A P L N L E I T V V S S E L T P S T N				71V-1658prt
801	A T T V P N V P G I P Y K A L V E R A G Y A P L N L E I T V V S S E L T P S T N				BFS-1703prt
	850	860	870	880	
841	K E Y V T C <u>K</u> F H T V I P S P Q V K C C G S L E C K A S S K A D Y T C R V F G G				71V-1658prt
841	K E Y V T C <u>R</u> F H T V I P S P Q V K C C G S L E C K A S S K A D Y T C R V F G G				BFS-1703prt

	890	900	910	920	
881	V Y P F M W G G A Q C F C D S E N T Q L S E A Y V E F A P D C T I D H A V A L K				71V-1658prt
881	V Y P F M W G G A Q C F C D S E N T Q L S E A Y V E F A P D C T I D H A V A L K				BFS-1703prt
	930	940	950	960	
921	V H T A A L K V G L R I V Y G N T T A H L D T F V N G V T P G S S R D L K V I A				71V-1658prt
921	V H T A A L K V G L R I V Y G N T T A H L D T F V N G V T P G S S R D L K V I A				BFS-1703prt
	970	980	990	1000	
961	G P I S A A F S P F D H K V V I R K G L V Y N Y D F P E Y G A M K P G A F G D I				71V-1658prt
961	G P I S A A F S P F D H K V V I R K G L V Y N Y D F P E Y G A M K P G A F G D I				BFS-1703prt
	1010	1020	1030	1040	
1001	Q A S S L D A T D I V A R T D I R L L K P S V K N I H V P Y T Q A V S G Y E M W				71V-1658prt
1001	Q A S S L D A T D I V A R T D I R L L K P S V K N I H V P Y T Q A V S G Y E M W				BFS-1703prt
	1050	1060	1070	1080	
1041	K N N S G R P L Q E T A P F G C K I E V E P L R A S N C A Y G H I P I S I D I P				71V-1658prt
1041	K N N S G R P L Q E T A P F G C K I E V E P L R A S N C A Y G H I P I S I D I P				BFS-1703prt
	1090	1100	1110	1120	
1081	D A A F V R S S E S P T I L E V S C T V A D C I Y S A D F G G S L T L Q Y K A D				71V-1658prt
1081	D A A F V R S S E S P T I L E V S C T V A D C I Y S A D F G G S L T L Q Y K A D				BFS-1703prt
	1130	1140	1150	1160	
1121	R E G H C P V H S H S T T A V L K E A T T H V T A V G S I T L H F S T S S P Q A				71V-1658prt
1121	R E G H C P V H S H S T T A V L K E A T T H V T A V G S I T L H F S T S S P Q A				BFS-1703prt
	1170	1180	1190	1200	
1161	N F I V S L C G K K S T C N A E C K P P A D H I I G E P H K V D Q E F Q A A V S				71V-1658prt
1161	N F I V S L C G K K T T C N A E C K P P A D H I I G E P H K V D Q E F Q A A V S				BFS-1703prt
	1210	1220	1230		
1201	K T S W N W L L A L F G G A S S L I V V G L I V L V C S S M L I N T R R .				71V-1658prt
1201	K T S W N W L L A L F G G A S S L I V V G L I V L V C S S M L I N T R R .				BFS-1703prt

Decoration 'Decoration #1': Box residues that differ from the Consensus.

Appendix C a) Sequence of the 5' end of WNSTA-3 (WEE 71V-1658) aligned with the 5' ends of HJ (HJV01trunc), EEE NA [EEENA5'(WEAV)] and EEE SA (EEESA01); and
b) Sequence of the 3' end of WNSTA-3 (WEE 71V-1658) aligned with the 5' end of WEE strain BFS 1703 (Weaver, *et al*, 1993) as described in text.

	10	20	30	40	
1	A T A G G G T	G T G G T G T A G A G G	A A C C T A C C C T A C	A A A C T A A T C	WNSTA3-M13RV
1	- - - - -	- - - - -	- - - - -	- - - - -	HJV01trunc
1	A T A G G G T A T G G T G T A G A G G C A	A C C A C C C T A T T T C C	- - A C C		EEENA5' (WEAV)
1	A T A G G G T A T G G T G T A G A G G C A	G C C A C C C G A C C T A T	- - - C C		EEESA01
	50	60	70	80	
41	G A T C C A A T A T G G A A A G A A T T C A C G T T G A C T T A G A T G C T G A				WNSTA3-M13RV
1	- - - - -	- - - - -	- - - - -	- - - - -	HJV01trunc
39	T A T C C A A A A T G G A G A A A G T T C A T G T T G A C T T A G A C G C A G A				EEENA5' (WEAV)
38	T A T C C A A A A T G G A G A A A G T A C A C G T A G A C T T A G A C G C T G A				EEESA01
	90	100	110	120	
81	C A G C C C G T A T G T C A A G T C G T T A C A G C G G A G C T T T C C A C A A				WNSTA3-M13RV
1	- - - - -	- - - - -	- - - - -	- - - - -	HJV01trunc
79	C A G C C C A T T C G T C A A G T C A C T G C A A A G A T G C T T T C C A C A T				EEENA5' (WEAV)
78	C A G C C C A T A C G T C A A G T C G C T G C A A A A G T G C T T T C C G C A T				EEESA01
	130	140	150	160	
121	T T T G A G A T C G A A G C A A G G C A G G T C A C T G A C A A T G A C C A T G				WNSTA3-M13RV
1	- - - - -	- - - - -	- - - - -	- - - - -	HJV01trunc
119	T T T G A G A T A G A A G C A A C G C A G G T C A C T G A C A A T G A C C A T G				EEENA5' (WEAV)
118	T T T G A G A T A G A A G C C A C G C A G G T C A C C G A C A A T G A C C A T G				EEESA01
	170	180	190	200	
161	C C A A T G C C A G A G C G T T T T C G C A T G T G G C A A C A A A G C T C A T				WNSTA3-M13RV
24	C T A A T G C C A G A G C G T T T T C G C A T G T G G C T A C A A A G C T C A T				HJV01trunc
159	C T A A T G C T A G G C G T T T T C G C A C C T A G C T A C T A A G C T C A T				EEENA5' (WEAV)
158	C T A A T G C T A G A G C G T T T T C G C A T C T G G C T A C A A A A C T C A T				EEESA01
	210	220	230	240	
201	T G A G A G C G A A G T C G A C C G G G A C C A A G T T A T C T T G G A C A T T				WNSTA3-M13RV
64	A G A G A G C G A A G T C G A C C G G G A G C A G A T T A T - - - - A C - - -				HJV01trunc
199	T G A G G G A G A A G T G G A T A C A G A C C A G G T G A T C C T G G A T A T T				EEENA5' (WEAV)
198	C G A A A G C G A A G T G G				EEESA01
	250				
241	G G A A G T G C G C C C				WNSTA3-M13RV
96	- - - - - T				HJV01trunc
239	G G G A G C G C G C C T				EEENA5' (WEAV)
211					EEESA01

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Decoration 'Decoration #2': Box residues that match the Consensus exactly.

		10	20	30	40	
1	G A	C C A C C A T T T C A C G A G T T T G C G T A C G A G A G T C T C A A G A	wnsta3t7-rc			
1	G A T C C A C C A T T T C A C G A G T T T G C G T A C G A G A G T C T C A A G A	WEU01065TRC				
		50	60	70	80	
41	C C G G A C C A G C A G C A C C T C A	N N	A A A G T C C C A A C C A T C G G A G	wnsta3t7-rc		
41	C A C G A C C A G C A G C A C C T C A C A A A		G T C C C A A C C A T C G G A G	WEU01065TRC		
		90	100	110	120	
81	T C T A T G G A G T G C C A G G T T C A G G T A A A T C T G G A A T C	N N	C A A	wnsta3t7-rc		
80	T C T A T G G A G T G C C A G G T T C A G G T A A A T C T G G A A T C A T C A A	WEU01065TRC				
		130	140	150	160	
121	A A G C G C T G T G A C T A A G A A A G A T C T G G T T G T G A G T G C G A A G	wnsta3t7-rc				
120	A A G C G C T G T G A C T A A G A A A G A T C T G G T T G T G A G T G C G A A G	WEU01065TRC				
		170	180	190	200	
161	A A G G A A A A C T G C G C A G A A A T	C	A T C A G G G A T G T A A G G A G G A	wnsta3t7-rc		
160	A A G G A A A A C T G C G C A G A A A T T A T C A G G G A T G T A A G G A G G A	WEU01065TRC				
		210	220	230	240	
201	T G A G A C G T A T G G A T G T T G C T G C T A G G A C T G T T G A T T C A G T	wnsta3t7-rc				
200	T G A G A C G T A T G G A T G T T G C T G C T A G G A C T G T T G A T T C A G T	WEU01065TRC				
		250	260	270	280	
241	G C T T C T A				wnsta3t7-rc	
240	G C T T C T A	A A T G G G G T T A A G C A C C C C G T T A A C A C C C T G T A C	WEU01065TRC			
247					wnsta3t7-rc	
280	A				WEU01065TRC	

Decoration 'Decoration #1': Box residues that differ from the Consensus.

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A 3100 bp cDNA clone (pcDW-12) of the 26S region of western equine encephalitis (WEE) virus strain 71V-1658 was identified by dot blot hybridization from a cDNA library. The missing 5' end was PCR-amplified engineered into pcDW-12 to obtain a full length clone of the 26S region. The resulting construct (XH-7) was restriction mapped and completely sequenced on both strands. Only eleven of the sixty-three nucleotide differences resulted in amino acid changes when the sequence was compared to WEE strain BFS-1703 (Hahn *et al*, 1988). In addition, the high degree of conservation of the structural proteins was maintained when compared to the N-terminal sequence of the E1 and E2 proteins of the McMillan strain of WEE (Bell *et al*, 1983). The conserved nature of the structural proteins would indicate that one strain of WEE should be able to cross-protect against all WEE strains. A 2.2 kb fragment at the 5' end of the genome was also cloned and sequenced, and demonstrated high homology to the available sequence for eastern equine encephalitis (EEE) and Highlands J (HJ) viruses, adding further evidence that the entire 5' nonstructural region of WEE was derived from EEE. In summary, the genetic cloning and sequencing of the WEE 26S region marks the critical first step in the generation of a subunit vaccine to WEE.

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